Dissection of the Interplay Between HPV E6 and Its Cellular and Viral Targets

Thesis

Dissection of the Interplay Between HPV E6 and Its Cellular and Viral Targets

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This thesis is submitted for the degree of Doctor of Philosophy in the Faculty of Life Sciences of the Open University, UK

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staršem, učiteljem mojega življenja in

Sandru, moji sreči

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In nenazadnje, hvala vsem mojim najdražjim brez katerih bi bilo to delo nemogoče ter mojima dvema kepicama, Miši in Fičotu.

Contents

List of Figures

Abstract

Dissection of the Interplay Between HPV E6 and its Cellular and Viral Targets

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Whilst numerous studies have assigned different activities to the Human Papillomavirus (HPV) proteins, most of these analyses have been performed using only individual viral proteins, in the absence of other viral gene products that would be encountered in the context of a normal viral infection. Because of this, we initiated a series of studies to investigate the biological consequences of coexpressing various combinations of the HPV-encoded proteins. We show that HPV E2 and E6 exert dramatic regulatory effects upon each other's activities, which are mediated by a direct protein-protein interaction. These include relocalisation and alteration of substrate specificity, as well as the fine tuning of viral DNA replication and gene expression. At the same time L2 was found to induce enhanced accumulation of E6 in promyeloitic leukemia oncogenic domains (PODs); one consequence of which is the targeting of the POD-associated protein Daxx for proteasome mediated degradation. These findings provide unique insights into the complexity of the viral life cycle, and suggest alternative models for the role of loss of E2 and potentially of L2 during malignant progression.

Using peptides isolated from an E6-specific library the significance of the E6/E6-AP interaction for E6 target degradation was also investigated. These studies reveal striking differences in the mechanism by which E6 targets its cellular substrates for degradation and provide compelling evidence for the role of E6-associated ubiquitin ligases other than E6-AP in the degradation of certain E6 targets. This has profound importance for studies aimed at developing therapeutics to target the E6/E6-AP interaction.

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Introduction

General definition and genome organisation

The papillomaviruses (papilla = nipple; oma = tumour) are a group of small DNA viruses which cause a spectrum of epithelial proliferative lesions ranging from warts to cancer. These viruses have two special properties which define their biology - species specificity and tissue tropism (Howley & Lowy, 2001). Within a species the individual viruses show a predilection for either cutaneous or mucosal surfaces and within the groups of skin or mucosal viruses they can be separated into "high" or "low" risk types depending upon their oncogenic potential (zur Hausen, 1996). The recognition that human papillomaviruses (HPVs) are an important cause of human cancer has heightened their medical importance and to date over 100 different viral types have been described; about one-third of these infect epithelial cells in the genital tract (de Villiers et al., 2004). Genital papillomavirus infections can be passed between individuals through sexual contact, and represent one of the most common sexually transmitted diseases (Wheeler et al., 2002). Some HPV types such as 6 and 11 induce only benign warts in the genital tract (condylomata acuminatum). They are referred to as low-risk types, as they induce lesions with a low risk of progression to malignancy. In contrast, the high-risk HPV types are associated with the development of anogenital cancers, including those of the cervix (zur Hausen, 1996; Howley & Lowy, 2001). Approximately 99% of cervical cancers contain HPV DNA of the high-risk types (Walboomers et al., 1999), with type HPV-16 being the most prevalent, followed by types 18, 31, 33, and 45 (zur Hausen, 1996; Howley & Lowy, 2001).

Human papillomaviruses (HPVs) are nonenveloped viruses with icosahedral capsids that replicate their genomes within the nuclei of infected host cells. The double-stranded, circular DNA genomes of all HPVs are approximately 8 kb in size. In virions, the HPV DNA is found associated with cellular histones to form chromatin-like complexes (Favre et al., 1977). The informational content of all the HPV genomes is organized into three major subregions as shown in Figure 1: the early region (E genes) encoding proteins required for viral replication

and regulation, the late region (L genes) encoding the two capsid proteins, and the noncoding region known as the upstream regulatory region (URR) (Androphy et al., 1987b). The early proteins (E) in high-risk HPV types are expressed from the early promoter (P97 in HPV-16 and 31, and P105 in HPV-18) prior to productive viral DNA replication (Rohlfs et al., 1991; Hummel et al., 1992; Sang & Barbosa, 1992), while the late proteins (L) are expressed from the late promoter, which consists of a number of initiation sites that map to sequences within the E7 ORF (Ozbun & Meyers, 1998; del Mar Pena & Laimins, 2001; Grassmann et al., 1996; Nilsson et al., 1996). The URR of HPVs shows remarkable functional conservation, as many transcription factor binding sites are present at similar locations. Promoter activity is likely to be mediated by complex protein-protein interactions, as well as by competition for binding. Elements important for early promoter activation are a TATA box, located approximately 30 nucleotides upstream of the transcript initiation site, and a binding site for the Sp1 transcription factor found upstream of the TATA box in the promoter proximal region (Gloss & Bernard, 1990). Binding of Sp1 to this site is believed to recruit the preinitiation complex to assemble at the early promoter. Furthermore, the URR of genital HPVs contains several binding sites for AP-1 (Chong et al., 1990; Thierry et al., 1992) and NF-1 (Gloss et al., 1989), which have been shown to play an important role in early promoter activity. Interestingly, all genital HPVs contain a conserved NF-1 site within two nucleotides of a binding site for Oct-1, and Oct-1 binding at this site appears to synergize with NF-1 activation (O'Connor & Bernard, 1995). In contrast Oct-1 seems to exert a repressive role on the early promoter in the absence of interaction with other factors (Hoppe-Seyler et al., 1991). Another negative regulator of HPV expression is the YY1 protein (Bauknecht et al., 1992) and, although the exact mechanism of YY1 repression has not been elucidated, it may act by inhibiting AP-1 transactivation (O'Connor et al., 1996). An important question in HPV biology is what determines the epithelial tropism of the virus. Numerous studies have demonstrated that the HPV enhancer activates expression preferentially in epithelial cells, and a potential explanation for this is that epithelial-specific factors activate enhancer function. One potential candidate is a POU domain protein

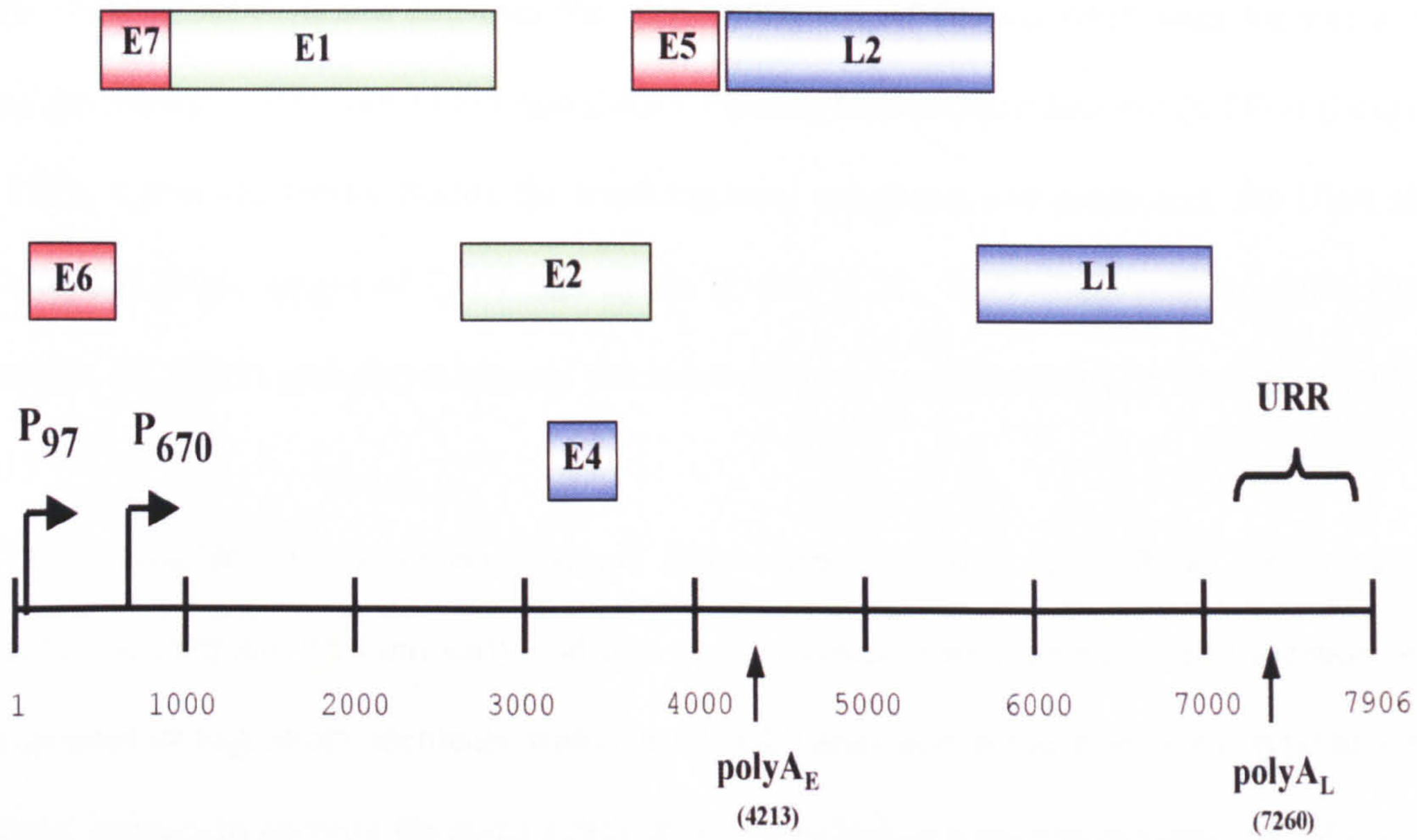


Figure 1. ***Genomic organisation of high-risk HPV-16.***
The circular genomes are represented in a linear form for convenience. The diagram indicates the ORFs of the early (E) and late (L) genes, the upstream regulatory region (URR), the two major promoters that drive viral gene expression (P_{97} and P_{670}) and the two polyadenylation sites (A_E4213 and A_L7260). The lower part of the figure shows the classification of viral ORFs according to their functions.

designated the epidermal octamer-binding factor 1 (Epoc-1), which is present exclusively in differentiated epithelium (Yukawa et al., 1996). Epoc-1 binds to the promoter-proximal region of the URR of HPV-18 and activates the early promoter. Additional candidates for imparting tissue specificity in HPV are TEF-1 and CAAT-binding transcription factor-1 (CTF-1) (Ishiji et al., 1992; Apt et al., 1993). Beside the transcriptional enhancers and promoters, the URR also contains the single origin of DNA replication (Ustav et al., 1991; DelVecchio et al., 1992; Chiang et al., 1992) and polyA signals for termination of transcription (Terhune et al., 1999, 2001).

All the coding regions occur on a single DNA strand, so only one strand serves as the transcription template for both early and late gene products. The number, precise location, and arrangement of regulatory elements within the URR varies somewhat from virus type to virus type and appears to account for some subtle differences in host-viral interactions (del Mar Pena & Laimins, 2002). In addition, other promoters and RNA splicing signals are variably located within the early coding region. The net result of these differences is complex RNA transcription patterns that are HPV type specific and which produce changes in some of the proteins expressed. Nonetheless, there is considerable conservation in the overall regulation of viral gene expression, and the major viral protein products are common to all HPVs.

HPV life cycle and viral gene expression

HPVs are exclusively epitheliotropic, and their replication is intimately linked to the differentiation process of the host keratinocytes as shown in Figure 2. Primary HPV infection is thought to occur in basal cells following penetration of the epithelium after wounding. The actual target cell of infection is believed to be some kind of primitive keratinocyte or stem cell (Stubenrauch & Laimins, 1999). The precise viral receptor(s) expressed on the surface of the basal keratinocytes remains undefined, but it is unlikely to be a unique molecule as numerous studies have shown binding and uptake of papillomavirus virions or pseudovirions in a wide variety of cell types (Roden et al., 1994; Muller et al., 1995; Volpers et al., 1995; Qi et al.,

1996). Experimental evidence supports two specific types of host cell molecules as receptors; alpha 6 integrin (Evander et al., 1997; McMillan et al, 1999; Yoon et al., 2001) and glycosaminoglycans such as heparin and heparin sulfate (Joyce et al., 1999; Giroglou et al., 2001; Combita et al., 2001). Although the role of each in a natural infection is still unclear, the heparin receptor binding could provide an initial low affinity binding followed by a higher affinity integrin receptor which would, in turn, mediate internalisation. Following entry, the viral genomes are established as episomes at approximately 50 copies per cell, which replicate in synchrony with the cellular DNA replication, until the infected basal cell enters the terminal differentiation pathway (Stubenrauch & Laimins, 1999; Flores & Lambert, 1997). At that point the virus life cycle switches to the "productive" phase in which the viral genome is amplified to a high copy number by a "rolling circle" mechanism (>10^5 copies per cell). At the same time, the capsid proteins are expressed and, ultimately, progeny virions are produced and released (Stubenrauch & Laimins, 1999; Flores & Lambert, 1997). Concomitantly, multiple papillomavirus gene products exert effects on the differentiating cells resulting in hyperproliferation and the histological changes characteristic of these infections (Howley & Lowy, 2001). It is this epithelial cell differentiation-dependent modulation of the viral transcriptional program that confers the profound species specificity upon these viruses and prevents their propagation in any simple cell culture system (Howley & Lowy, 2001). Hence, the molecular events that regulate the productive stages of HPV infection are not well established. However, our understanding has been significantly enhanced by the use of organotypic culture systems (Frattini et al., 1997; Stanley, 2002), xenotransplantation (Ozbun & Meyers, 1997), and the use of animal papillomaviruses that cause lesions similar to those found in humans (Peh et al., 2002); all of which allow the propagation of the virus and the study of late gene expression. Using these methods new work has begun to make an impact on this area, and a basic picture of events is now emerging. The analysis of lesions caused by different papillomavirus types has revealed that the organization of the viral life cycle is common to many different HPV types (Peh et al., 2002), where HPV gene expression follows a highly

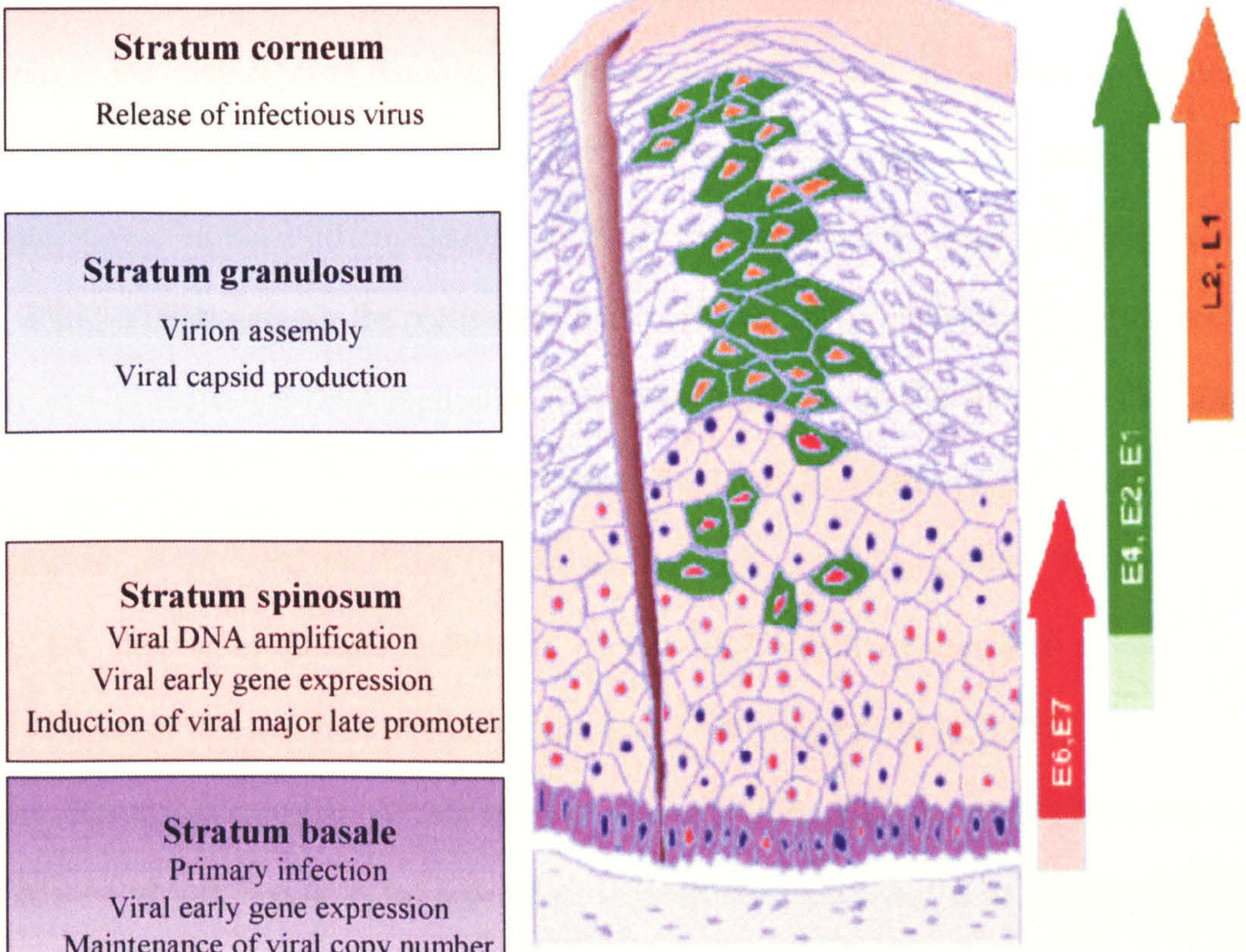


Figure 2. ***Schematic representation of the interrelationship between epithelial differentiation and the HPV-life cyc***le, indicating the stages at which the expression of different viral proteins occurs. (Courtesy of J. Doorbar).

ordered sequence, with individual gene products appearing at specific points during the differentiation of the infected keratinocyte (Howley & Lowy, 2001; Peh et al., 2002). This is summarised in Figure 2 where it can be seen that the early proteins (E6 and E7) first appear in basal/parabasal cells (Peh et al., 2002). The function of these proteins is to maintain the HPV episomes in the basal layer of the skin (Thomas et al., 1999a; Oh et al., 2004b), and to modify the cellular environment to facilitate viral DNA replication in a cell which has left the basal layer and started terminal differentiation and which has consequently exited the cell cycle (Pim et al., 2001). Simultaneously the replication factors E1 and E2 are expressed and act to regulate viral gene expression and DNA replication (Liu & Melendy, 2002). The functions of the E4 and E5 proteins are not yet fully understood; however, they both have been proposed to be involved in regulation of late viral functions (Peh et al., 2004; Genther et al., 2003; Fehrmann et al., 2003). E4 expression follows activation of the differentiation-dependent promoter upon differentiation of infected keratinocyte (Chow et a., 1987; Nasseri et al., 1987). Its appearance has been reported to coincide with the onset of vegetative viral genome amplification and marks the initiation of late events in the suprabasal spinous layer (Doorbar et al., 1997). Only upon further differentiation, in the upper spinous layers, are the capsid proteins expressed, allowing the assembly of infectious virions and their release from the epithelial surface (Peh et al., 2002) (Figure 2).

It is still not clear how the differentiation program of the host cell is able to activate the productive life cycle of HPV. The most likely mechanism centers on the activation of the late viral promoter, resulting in high-level expression of transcripts encoding the viral replication proteins, E1 and E2, along with the late genes. Unlike the early promoter, the late promoter is not negatively regulated by E2 protein, and high levels of expression occur upon differentiation, leading to amplification of viral DNA (Steger & Corbach, 1997). This increase in template numbers results in a further increase in expression of the replication proteins. However, it is possible that cellular or other viral factors are deregulated upon differentiation and that these factors also contribute to activation of late viral functions, but their identification is only at the

beginning.

HPV infection, cancer and therapy

Infection with human papillomaviruses can lead to both acute disease and long-term persistence of the viral genome, and infections have been associated with three clinical states: latent or unapparent infections, clinical warts, and certain cancers, particularly cervical carcinoma (zur Hausen, 1996; Burd, 2003). Cervical cancer is one of the most common cancers among women worldwide, with about 470,000 newly diagnosed cases and an estimated 230,000 deaths per year. The greatest burden of cervical cancer is in developing countries where it is often the most common female malignancy (Parkin et al., 2000; Wheeler et al., 2002). Epidemiological evidence has convincingly demonstrated that infection with HPV is the greatest risk factor (Bosch et al., 1995), and its role in the progression of the precursor lesions to cervical cancer has been well established (Bosch et al., 2002). In contrast to the causal role of high-risk HPVs in the origin of cervical cancer, less is known about the cutaneous HPV types. The association between HPV and non-melanoma skin cancers (NMSC) was first identified in patients with *Epidermodysplasia verruciformis* (EV), and later in recipients of organ transplants, where these patients develop NMSC at sun-exposed sites. Again, a subset of virus types, in particular HPV-5 and HPV-8, is associated with the development of these malignancies (Fuchs & Pfister, 1996; Benton & Arends, 1996). One interesting feature of these cancers is that the viral DNA is present in less than one copy per cell (Howley & Lowy, 2001). This finding suggests that cutaneous HPV types may be part of a "hit and run" mechanism of tumour induction, being important for tumour initiation and progression, but not for the maintenance of the malignant phenotype.

There are two important features in the HPV life cycle that indirectly contribute to the development of cancer. First, the replicative phase of HPV is confined to differentiating epithelial cells that have exited the cell cycle and which are normally non-permissive for DNA synthesis (Doorbar et al., 1997). Since HPVs use cellular enzymes to replicate their genomes,

they need to induce the cellular replication machinery, while simultaneously maintaining differentiation. This is achieved by the combined activity of the viral E6 and E7 oncoproteins. However, should this process be in any way perturbed, then events leading to cell immortalisation and malignancy can be initiated. This has been amply demonstrated in *in vitro* assays, where E6 and E7 can efficiently cooperate to immortalize human keratinocytes (Munger et al., 1989; Hawley-Nelson et al., 1989; Barbosa & Schlegel, 1989). Additionally, the spatial and temporal differences between high and low-risk HPVs with respect to their sites of DNA replication within the epithelium are also likely to be critical. Low-risk HPVs tend to initiate DNA replication in the less differentiated cell population where elements of the cellular DNA replication machinery are still present. In contrast, high-risk HPVs replicate in the higher levels of the epithelium, and therefore require more vigorous priming of the cell division machinery (Doorbar et al., 1997). These differences are very clearly demonstrated in the respective biochemical activities of the high and low-risk HPV E6 and E7 proteins (see below).

The initiation of the events which ultimately result in malignancy is still poorly understood. However, a key point to remember is that this always entails the loss of replicative competence for the virus and, as such, is not part of the viral life cycle. Indeed, during this process the viral DNA frequently becomes integrated into the host DNA. This is often accompanied by large deletions in the viral DNA and is coupled with uncontrolled expression of the E6 and E7 oncoproteins (Yee et al., 1985; Schwarz et al., 1985; Baker et al., 1987), which then continue to drive the malignant progression of the disease (Munger et al., 1989; Howley-Nelson et al., 1989; Barbosa & Schlegel, 1989). Figure 3 shows in a schematic manner some of the major components of the transition from HPV infection to cervical cancer. Initial infection with the high-risk types may cause low grade disease (low-grade squamous intraepithelial lesions, LSIL), which is manifested by inhibition of the normal differentiation in the lower third of the epithelium. The lesion may remain low-grade, may regress, or may progress to severe dysplasia or HSIL (high-grade squamous intraepithelial lesions). This latter stage may persist or may start to invade below the basement membrane leading to metastatic disease (McMurray et al., 2001).

Control of cervical cancer through well-conducted screening programs has largely failed in developing countries where the disease is still a leading cause of cancer death. It is thus a major goal to develop safe and effective therapeutics to prevent and to treat HPV infections and their associated diseases. Most of the work to date has focused on the development of simple effective vaccines against HPVs; however, the existence of chronic HPV infections suggests that natural immunity is not always fully effective in controlling HPV infection (Steele, 2001; Breitburd & Coursaget, 1999). Therefore, an enormous and growing population of infected individuals exists that would benefit from papillomavirus-specific therapy. Unfortunately, no specific anti-papillomavirus agents are available so current therapy for these infections is directed primarily at physical destruction of infected tissues, with inherent associated risks to the patient (Baker & Tyring, 1997). Less destructive, more reliable, and more specific treatments would be desirable, but remain elusive.

Functions of the viral proteins

Regulatory proteins

The viral regulatory proteins encompass those proteins that control gene expression and replication of the viral genome. For papillomaviruses these functions are performed primarily by the E1 and E2 proteins, each of which exhibit roles in genome DNA replication and in the case of E2, also in transcriptional regulation. Both E1 and E2 are site-specific DNA binding proteins that recognise *cis* elements in the viral genome and form complexes with each other (Liu & Melendy, 2002). These are the sole viral proteins required for viral DNA replication *in vitro* (Kuo et al., 1994) and in transient assays *in vivo* (DelVecchio et al., 1992; Chiang et al., 1992).

E1 proteins

The largest ORF in the papillomavirus genome encodes the origin specific DNA binding protein E1. This is the most conserved papillomavirus protein and the only one endowed with

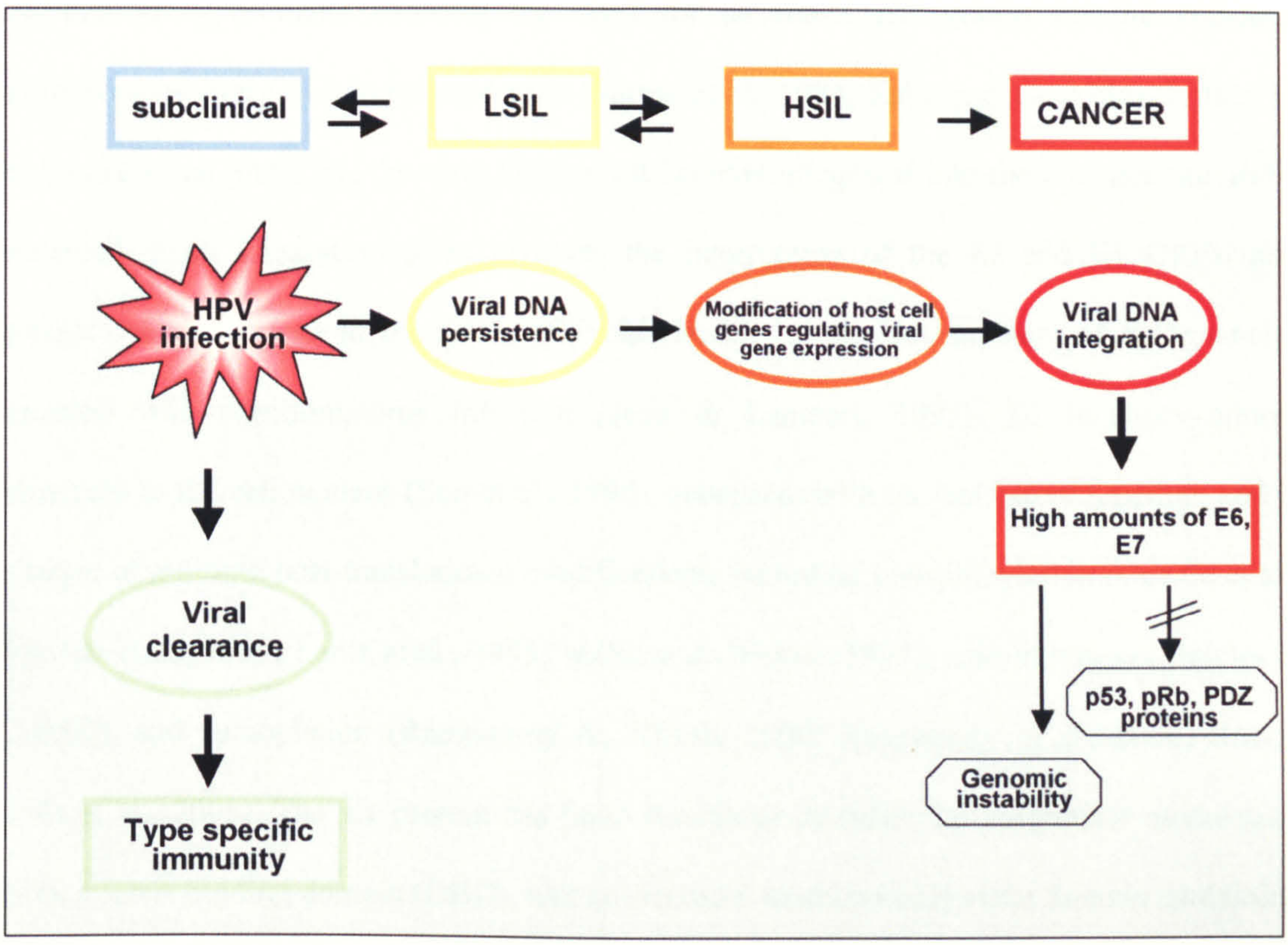


Figure 3. ***Mechanisms of HPV carcinogenesis.***
Malignant progression is closely related to the persistence of viral DNA, its integration into cellular DNA, disruption of E2 ORF, and ultimately increased expression of E6 and E7 oncogenes, which degrade p53 and pRb, respectively, contributing to cell transformation. HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion. (Modified from zur Hausen, 1999).

enzymatic helicase activity (Yang et al., 1993). Based on its homology with the SV40 large T antigen, E1 was predicted to have a similar role to the one previously characterized for the SV40 protein during DNA viral replication (Clertant & Seif, 1984). This prediction was rapidly confirmed in experiments showing the need for an intact E1 protein for the episomal maintenance of viral DNA in infected cells (Sarver et al., 1984; Rabson et al., 1986). In fact, in the absence of an intact E1, the viral DNA must become integrated into the host genome to be maintained. Such integration often involves the interruption of the E2 and E1 ORFs, and represents an early stage in the series of events leading to the development of malignancies associated with papillomavirus infection (Jeon & Lambert, 1995). E1 localises almost exclusively to the cell nucleus (Sun et al., 1990), consistent with its replicative function, and is the target of multiple post-translational modifications, including phosphorylation (Cueille et al., 1998; Ma et al., 1999; Lentz et al., 1993; McShan & Wilson, 1997), ubiquitination (Malcles et al., 2002), and sumoylation (Rangasamy & Wilson, 2000; Rangasamy et al., 2000; Rosas-Acosta et al., 2005). The E1 protein has three functional domains: an N-terminal modulatory region, a DNA binding domain (DBD), and conserved C-terminal enzymatic domain containing the E2 binding site, and ATPase and helicase activities (Liu & Melendy, 2002). Current data indicate that the initiation of viral DNA replication involves a series of complex events that can be grouped into three consecutive stages: the initial binding and association of E1 to the origin of replication, the formation of the hexameric ATP-ase helicase complex, and the assembly of a replication complex via the recruitment of cellular DNA replication factors by the hexameric helicase complex (Liu & Melendy, 2002). In the first stage E1 binds as a dimer to an imperfect 18 bp palindromic sequence in the origin of replication (Holt et al., 1994; Holt & Wilson, 1995). The specific binding of E1 to this sequence is known to depend on E2 as the combined E1-E2 complex is several hundred times more sequence-specific than E1 alone (Sedman & Stenlund, 1995). Thus, E2 has traditionally been postulated to tether E1 specifically to the origin, which is located adjacent to an E2 binding motif on the viral DNA. However, this role has been recently re-evaluated based on extensive footprinting and cross-linking analyses showing that the DBD

region of E1 exhibits identical DNA binding properties to those observed for full-length E1 in combination with E2. This indicates that E1 may exhibit two different DNA binding activities: a relatively unspecific binding, displayed by the full-length molecule, and a highly specific binding activity, mediated by the DBD. According to this model, the role of E2 is then to unmask the highly selective E1 DBD-binding activity by interacting with the helicase domain of E1 (Stenlund, 2003). Upon E1 binding to E2 at the origin, the E1-E2 interaction and their respective association with the DNA leads to the formation of a significant bend in the DNA, which starts the melting of the viral origin. However, full melting requires the formation of the hexameric complex (Chen & Stenlund, 2002), which is the second stage during the initiation of viral replication, and requires the dissociation of E2 from E1 (White et al., 2001; Sanders & Stenlund, 1998). In the final stages of the initiation of viral DNA replication the cellular replication factors, the replication protein A (RPA) and DNA polymerase alpha-primase (polprim), are recruited to the origin by the E1 protein (Loo & Melendy, 2004; Masterson et al., 1998; Conger et al., 1999; Han et al., 1999). This step leads to the formation of a fully active replication complex. Besides the replication proteins recruited by E1 to the replication complex, several additional host cellular proteins have been found to bind E1, including the chaperones Hsp70 and Hsp40 (Lin et al., 2002; Liu et al., 1998), the cell cycle regulator cyclin E-Cdk2 kinase (Cueille et al., 1998; Ma et al., 1999), histone H1 (Swindle & Engler, 1998), hSNF5 (Lee et al., 1999), and Ubc9 (Rangasamy & Wilson, 2000). Although the physiological significance of some of these interactions remains to be determined, several appear to be quite interesting. Thus, the interaction of E1 with cyclin E-Cdk2 and its phosphorylation by this cell cycle regulated kinase is of pivotal relevance for the regulation of E1 replicative function (Ma et al., 1999; Lin et al., 2000). In addition, E1 was found to be sumoylated in cells, apparently due to its interaction with the ubiquitin-conjugating enzyme, Ubc9. This modification is required for the intranuclear accumulation of E1, and is therefore required for efficient origin-dependent replication (Rangasamy et al., 2000).

E2 proteins

The full-length E2 protein plays an important role in both viral DNA replication (as noted above) and transcription modulation. Papillomavirus E2 proteins have two highly conserved domains separated by an internal hinge region: a DNA-binding domain is located in the carboxy-terminal region of the protein and a transactivacting domain is located within the amino-terminal half of the protein (Giri & Yaniv, 1988). X-ray crystal structures are available for both the N- (Antson et al., 2000; Harris & Botchan, 1999) and C-terminal domains (Hegde et al., 1992; Hegde & Androphy, 1998; Kim et al., 2000), but crystallisation of the complete protein has not yet been achieved. The N-terminal domain has transcriptional transactivation function (Bouvard et al., 1994b), though this domain is much more structurally constrained than typical transactivators. E2 proteins from the high-risk types have stronger transactivational character than the low-risk types, but the significance of this difference in their life cycle is unknown (Kovelman et al., 1996). Between the transactivation domain and the C-terminal DNA binding domain lies the hinge region, which is less structured and less well conserved so this was initially believed to be an inner spacer. Subsequent studies have demonstrated a role in nuclear localisation and nuclear matrix attachment (Zou et al., 2000), and it is now clear that this region also makes functional contributions to E2 transcriptional activities (Steger et al., 2002; Lai et al., 1999). The C-terminal conserved domain of E2 functions in site-specific DNA binding and in dimerization (Bedrosian & Bastia, 1990; Prakash et al., 1992). This domain has a unique dimeric β-barrel structure that constitutes a novel type of DNA binding motif (Hegde et al., 1992; Hegde & Androphy, 1998). All E2 proteins recognise and bind a consensus sequence, $ACCN_6GGT$, where the four central nucleotides are A/T rich (Androphy et al., 1987b). Binding of dimeric E2 to this target element (called the E2BS) induces the bending of DNA around the protein (Bedrosian & Bastia, 1990; Thain et al., 1997) which may facilitate DNA looping for the juxtaposition of widely spaced transcriptional factors (Antson et al., 2000). The BPV-1 E2 transactivator can bind to seventeen sites scattered throughout the genome and it functions as the major activator of BPV-1 early gene expression (Li et al., 1989). In contrast, only four E2

binding sites are present in the URR of genital papillomaviruses (Liu & Melendy, 2002). Reporter assays involving the URR of HPV-16 and 18 revealed that E2 activates expression at low concentration, yet represses it when expressed at high levels (Steger & Corbach, 1997). A possible mechanism for this repression is through displacement of TBP and Sp1 when E2 binds to binding sites 3 and 4 (Tan et al., 1992; Dong et al., 1994), as illustrated in Figure 4. At low doses of E2 a weak activation was reported, probably through binding to the E2 binding site 1 (Bouvard et al., 1994b; Steger & Corbach, 1997).

In addition to its role as a transcriptional regulator, E2 is critical for viral DNA replication (Chiang et al., 1992; DelVecchio et al., 1992), where it acts as a loading factor to facilitate assembly of E1 on the viral origin as shown in Figure 4 (Sanders & Stenlund, 2000; Storey et al., 1995; Yasugi et al., 1997, Muller & Sapp, 1996). Subsequently, E2 is released as the E1 helicase assembles and DNA synthesis is initiated (Sanders & Stenlund, 1998; Liu et al., 1995). An intriguing recent study found that HPV-16 E2 actually had very little transcriptional effect on native, episomal HPV-16 DNA with an authentic chromatin organization, suggesting that E2's replication function may be of primary importance (Bechtold et al., 2003). This is supported by an earlier study which indicated that the transactivation activity of E2 is not essential for the stable maintenance of viral episomes, for the expression of early or late genes, nor for the differentiation-dependent genome amplification (Stubenrauch et al., 1998).

A second contribution of E2 to stable viral maintenance involves the tethering of viral genomes to host cell chromatin, possibly to ensure adequate partitioning of the viral genomes during cell division. Initial studies with BPV-1 indicated that E2 binding sites are required for stable episomal maintenance (Piirsoo et al., 1996). Subsequent studies found that E2 is associated with host chromatin via its transactivation domain (Skiadopoulos & McBride, 1998; Lehman & Botchan, 1998; Ilves et al., 1999). The transactivation domain is necessary and sufficient for chromatin association, and there is no requirement for the E2 DNA binding domain (Bastien & McBride, 2000). Furthermore, a recent study showed that the bromodomain protein Brd4 binds the N-terminal domain of E2 and functions as the major cellular receptor for tethering the viral

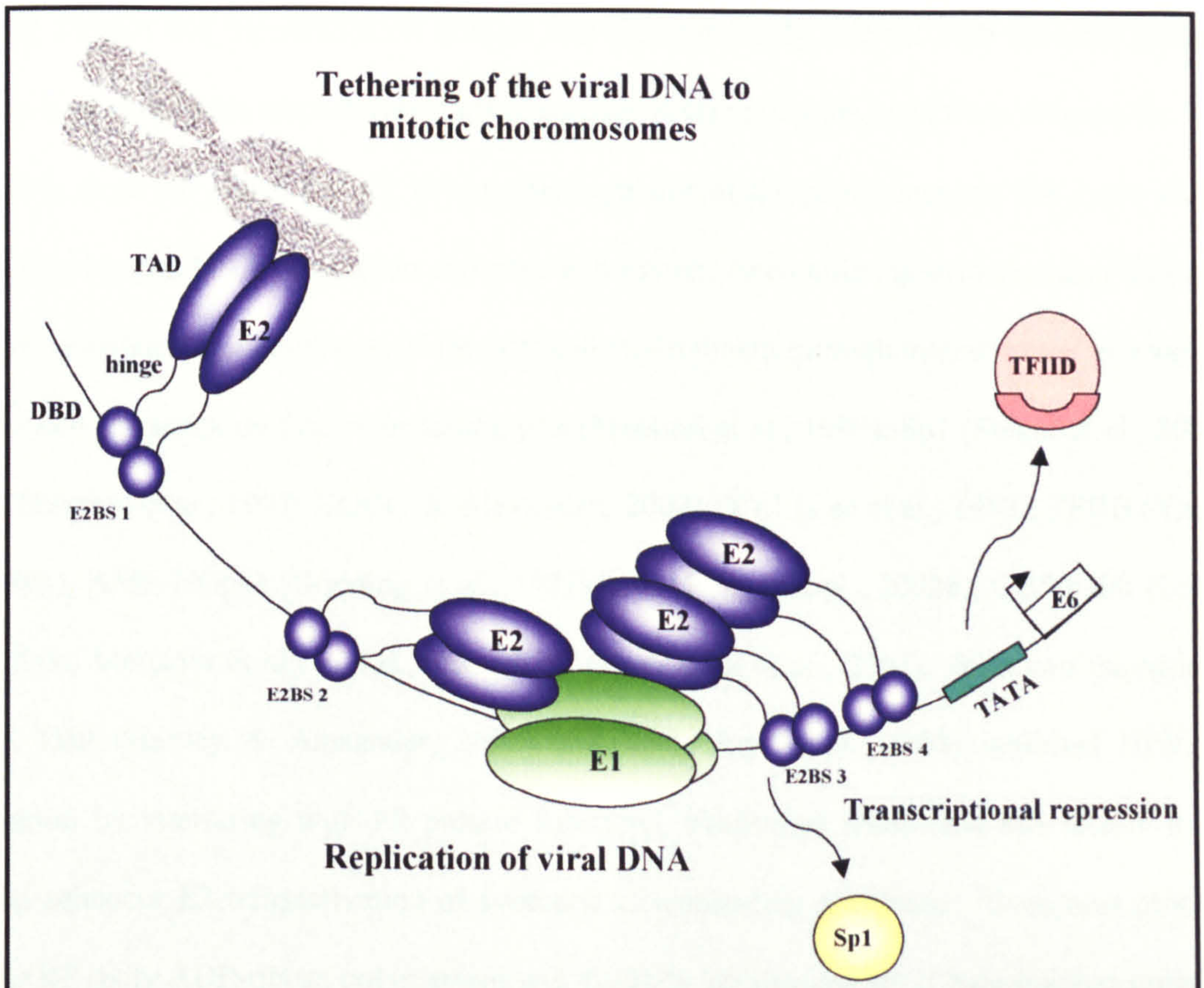


Figure 4. ***A schematic representation of the roles of the E2 protein showing the molecular consequences of E2 binding to its consensus binding sites in the URR.***

In binding to the E2BS 3 and 4, E2 represses transcription of early viral genes by interfering with the binding of transacription factors TFIID and Sp1 to their recognition sequences. E2 also acts as a loading factor to facilitate the assembly of E1 onto the viral origin via its ability to form protein-protein interactions with E1. Stimulation of the E1-DNA binding requires both the DNA binding activity of E2 and the presence of functional E2 binding sites in the origin (E2BS 2, 3, and 4). The third function of E2 is only schematically presented since it is not known to which E2BS E2 binds in tethering viral genomes to host cell chromatin. In a currently existing model, the E2 DNA binding (DBD) and transactivaction domains (TAD) can simultaneously interact with the viral genome and the host chromatin, respectively, thus physically juxtaposing the two DNAs (Modified from Blachon & Demeret, 2003).

genome onto mitotic chromosomes (You et al., 2004). This led to a model in which the E2 DNA binding domain and transactivation domain can simultaneously interact with the viral genome and the host chromatin, respectively, thus physically juxtaposing the two DNAs (Figure 4).

It is clear from the above that E2 is a master regulator of the papillomavirus life cycle with a direct role in viral DNA replication and gene expression. Accumulating evidence also indicates that E2 contributes to modulation of the host cell environment through interaction with a variety of host cell transcription factors including p53 (Massimi et al., 1999), Sp1 (Steger et al., 2002), TBP (Massimi et al., 1997; Hartley & Alexander., 2002), YY1 (Lee et al., 1998), TFIIB (Yao et al., 1998), AMF-1/Gps2 (Breiding et al., 1997), p/CAF (Lee et al., 2002b), CBP/p300 (Lee et al., 2000a; Marcello et al., 2000), and C/EBP (Hadaschik et al., 2003). With two exceptions, where TBP (Hartley & Alexander, 2002) and YY1 (Lee et al., 1998) inhibited HPV *ori* replication by interfering with E2 protein functions, binding of these host cell factors to E2 protein enhances E2-transactivation of synthetic E2-dependent promoters. Even host proteins like PARP (poly ADP-ribose polymerase) and TopBP1 (topoisomerase II beta-binding protein), whose main functions appear to be in other cellular processes, can bind E2 and function as transcriptional co-activators (Lee et al., 2002c; Boner et al., 2002). More importantly, authentic host cell promoters lacking E2 binding sites, including p21 (Steger et al., 2002), involucrin (Hadaschik et al., 2003), and hTERT (Lee et al., 2002a) are regulated by E2 in concert with the interacting co-activators. As expression of some of these co-activators has been shown to be differentiation-dependent, these transcriptional results are consistent with E2 not only regulating viral promoters, but also influencing host cell gene expression during epithelial differentiation.

E2 protein levels were also found to vary throughout the cell cycle, and one of the mechanisms of regulation is through proteasome-mediated degradation (Bellanger et al., 2001). For BPV E2, phosphorylation in the hinge region is required for subsequent ubiquitination (Penrose & McBride, 2000; McBride et al., 1989; Lehman et al., 1997). HPV E2 proteins have been also shown to be phosphorylated (Sanders et al., 1995) although the sites have not been identified, and whilst HPV E2 is ubiquitinated, this does not seem to require the hinge region (Bellanger et

al., 2001).

Finally, we should also consider the other properties of E2 when overexpressed in cervical carcinoma cells: that is induction of both G1 cell cycle arrest and cell death by apoptosis (Blachon & Demeret, 2003). The re-expression of E2 proteins in HPV-positive, but not HPV-negative, cervical cancer cells results in a G1 cell cycle arrest (Hwang et al., 1993; Dowhanick et al., 1995; Desaintes et al., 1997). E2-mediated G1 growth arrest requires E6/E7 promoter repression by E2 and is followed by the induction of cellular senescence (Wells et al., 2000; Francis et al., 2000; Goodwin & DiMaio, 2000, 2001). E2 has also been shown to induce apoptosis in both HPV-positive and HPV-negative cancer cell lines through both p53 dependent and independent mechanisms (Desaintes et al., 1997; Webster et al., 2000; Demeret et al., 2003).

In cervical carcinomas, high-risk HPV genomes are often found integrated into the cellular host DNA (Schwarz et al., 1985; Stoler et al., 1992). HPV DNA integration has been suggested to be an important event in the development of cervical cancer, since this often results in a disruption of the E2 region (Schwarz et al., 1985; Baker et al., 1987). Integration thus abrogates the inhibitory actions of the E2 proteins on the viral promoter of the E6 and E7 genes. This results in high-level expression of E6 and E7, and probably contributes to cellular transformation that eventually results in cancer (Schneider-Gadicke & Schwartz, 1986; Smotkin & Wettstein, 1986; Androphy et al., 1987a; Banks et al., 1987b; Jeon et al., 1995).

Transforming proteins

A major impetus for the study of the HPVs is their causal role in the development of cervical cancer and possibly other mucocutaneous malignancies (Howley & Lowy, 2001). Consequently, the focus on viral transforming proteins has been intense, culminating in the identification of the viral E5, E6, and E7 gene products as the mediators of virus-induced cellular transformation (Pim et al., 2001). It is now known that high-risk HPV E6 and E7 are the major viral oncoproteins and their continued expression is essential for maintenance of the transformed

phenotype (Smotkin & Wettstein, 1986; Androphy et al., 1987a; Banks et al., 1987b). The first evidence that HPV harboured oncogenic activity was observed in established rodent cells, where the HPV-16 E7 gene was found to efficiently induce malignant transformation (Kanda et al., 1988; Vousden et al., 1988). Numerous other studies have now shown that both E6 and E7 have intrinsic transforming activity. Both will cooperate with an activated *ras* oncogene in the transformation of primary rodent cells (Storey et al., 1988; Storey & Banks, 1993), and both exhibit transforming potential in primary mammary epithelial cells (Wazer et al., 1995). However the most relevant assay system, with respect to the natural target cells of the virus, is immortalisation of primary human cervical keratinocytes. These cells are notoriously difficult to immortalise, yet E6 and E7 will cooperate to bring about the immortalisation of this cell type (Durst et al., 1987; Woodworth et al., 1989; Munger et al., 1989; Hawley-Nelson et al., 1989). It should be emphasised that a key feature of these assays is that both E6 and E7 are required for this to occur, implying that the two viral oncoproteins normally cooperate in the processes of cell immortalisation *in vivo*. Another aspect of these assays is that these cells are not fully transformed. Additional genetic insults, achieved either through extended passage in culture, or by addition of activated oncogenes (Durst et al., 1989; DiPaolo et al., 1989), are required before these cells become fully transformed, thus highlighting the multistep nature of the disease process. In contrast to oncogenic HPV types, the E6 and E7 proteins from low-risk HPV types are unable to immortalise keratinocytes *in vitro* though they may moderately extend their life span (Thomas et al., 2001a).

By using transgenic mice expressing HPV-16 E6 or E7 from the human keratin 14 promoter, it has been possible to dissect the contribution of the two oncoproteins in the development of skin and cervical cancer (Herber et al., 1996; Song et al., 1999, 2000; Riley et al., 2003). In the cervix, E7 was found to cooperate with estrogen to induce both high grade dysplasia and invasive cervical malignancies. Although E6, in the absence of E7, was able to produce only low grade cervical dysplasia, the two viral oncoproteins together gave rise to a dramatic increase in the number of large, frank cervical cancers, indicating a role for E6 in malignant

progression (Riley et al., 2003). In the skin of the same mice, E7 induces primarily benign papillomas, whilst E6 produces fewer lesions, however those which do arise are more transformed (Herber et al., 1996; Song et al., 1999, 2000). Once again, the combination of E6 and E7 gives rise to more tumours of a more malignant phenotype, being concordant with a role for E6 as a late-stage malignant progression factor in both the cervix and the skin of double E6:E7 transgenic mice. Although much less is known about the cutaneous HPV E6 and E7 proteins, a recent study has shown that the high risk HPV-38 E6 and E7 proteins can substantially increase the life span of primary human keratinocytes (Caldeira et al., 2003). This is likely to have an important impact on studies aiming to establish a causal relationship between the presence of specific HPV types and skin cancers.

Activities of the E5 protein

The HPV E5 protein is considered a weak oncogene given its ability to transform mouse fibroblasts and keratinocytes, to cause the mitogenic stimulation of human keratinocytes, and to cooperate with E7 to stimulate proliferation of human keratinocytes (Leechanachai et al., 1992; Leptak et al., 1991; Pim et al., 1992; Straight et al., 1993; Bouvard et al., 1994a). HPV E5 proteins are small (~90 amino acids), extremely hydrophobic, and are located mainly at the endosomal membranes, Golgi apparatus, and, to a lesser extent, the plasma membranes (Conrad et al., 1993). In contrast to BPV-1 E5, which has been shown to encode the primary transforming function of the virus (Schlegel et al., 1986; Martin et al., 1989), little is known about the biological activity of HPV E5 (DiMaio & Mattoon, 2001). Much attention has focused on the ability of HPV E5 proteins to affect cellular growth factor receptors and the vacuolar ATPase (vATPare) since these are well characterized roles for the BPV E5 protein (Petti et al., 1991; Goldstein & Schlegel, 1990). HPV E5 preferentially binds to the epidermal growth factor receptor (EGFR) and enhances mitogenic signaling in a ligand-dependent manner (Pim et al., 1992; Hwang et al., 1995; Bouvard et al., 1994a). This effect is mediated through increased recycling of the receptor to the cell surface (Straight et al., 1993, 1995; Crusius et al., 1997),

which appears to be related to the ability of HPV E5 to bind the vATPase (Conrad et al., 1993), resulting in inhibition of acidification of endosomes (Straight et al., 1995; Adam et al., 2000; Briggs et al., 2001). However, all of these observations are called into question by studies in organotypic raft cultures (Fehrmann et al., 2003; Mayer & Meyers, 1998; Genther et al., 2003). In these studies there was neither colocalisation of E5 with EGFR (Mayer & Meyers, 1998) nor an E5-dependent change in EGFR levels (Fehrmann et al., 2003), and the overall conclusion of all three studies was that E5 exerted little if any detectable affect on EGFR under these conditions. Interestingly, E5 in these studies was found to have an important activity in regulating genome amplification and late gene expression in differentiated suprabasal cells (Fehrmann et al., 2003; Genther et al., 2003). Based on these data, a new function was proposed by the authors, suggesting that the E5 protein acts in the late phase of the viral life cycle, most likely in conjunction with E7, to support cell cycle progression.

Given the fact that E5 interferes with the functions of the Golgi apparatus and endosomes (Schapiro et al., 2000; Straight et al., 1995), it was predicted that not only endocytic cellular traffic (Thomsen et al., 2000) but also exocytic transport would be disrupted in E5-expressing cells, including transport of the MHC I complex. Indeed, BPV E5 proteins induce downregulation of MHC I (Ashrafi et al., 2002). Lack of surface MHC I is observed also in cells expressing HPV E5 proteins (Venuti & Campo, 2002), but how E5 achieves downregulation of MHC I is not yet known. E5-induced loss of surface MHC I expression in the infected basal cell would prevent presentation of viral antigen to effector T-cells and would thus contribute to the evasion of immune surveillance.

Another function for E5 is suggested by the recent observations that HPV-16 E5 protects cells from the apoptosis induced by UV-B irradiation (Zhang et al., 2002), and by the TRAIL and FasL ligands (Kabsch & Alonso, 2002). The E5-mediated protection from UV-B occurred via enhancement of the P13K-Akt and ERK1/2MAPK signaling pathways rather than through any effect on p53 such as that exerted by E6 (see below). Thus, E5 and E6 have at least additive and possibly synergistic roles in abrogating apoptosis.

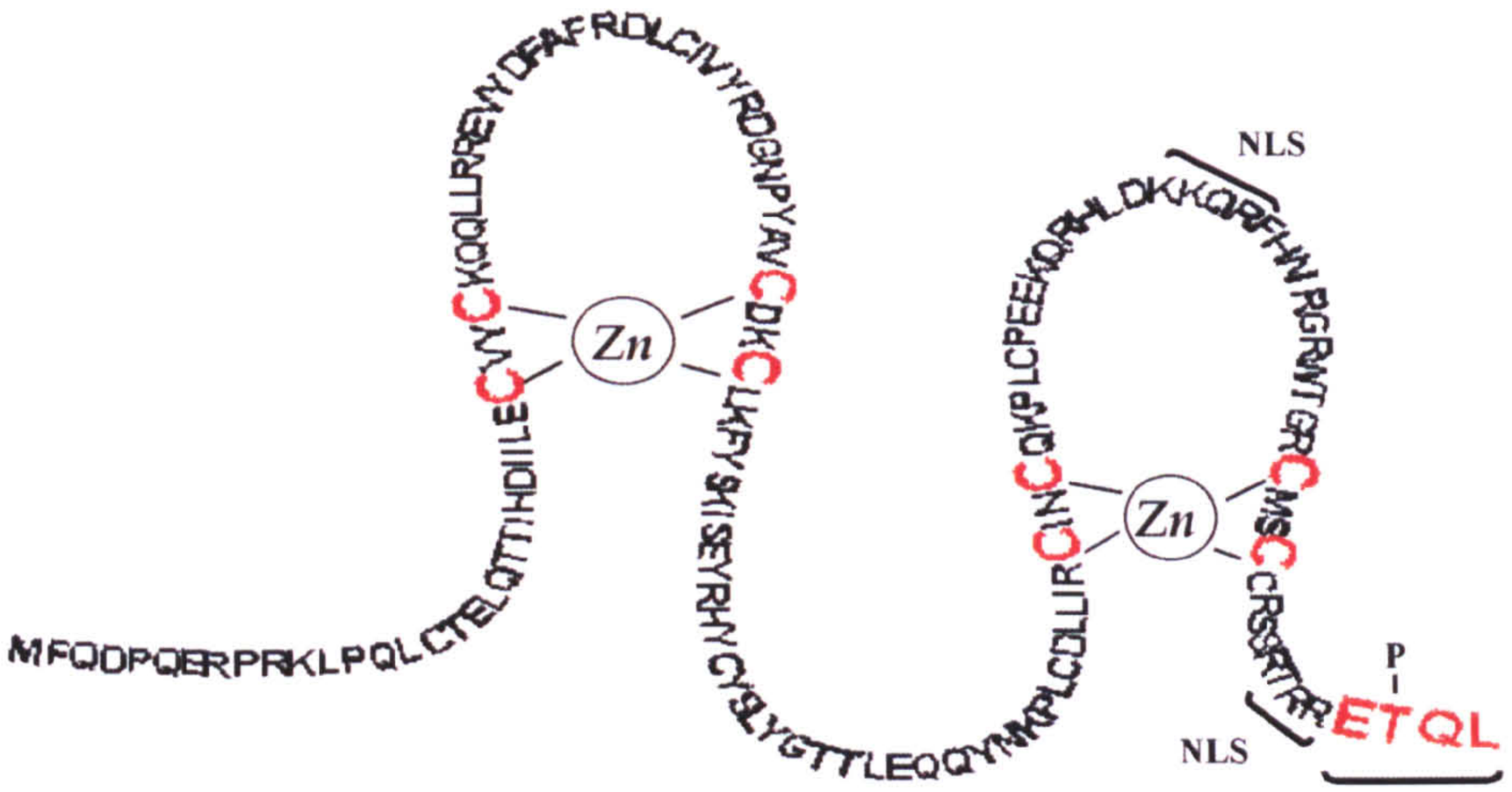
Activities of the E6 protein

The HPV E6 proteins are small polypeptides of approximately 150 amino acids, and have four Cys-X-X-Cys motifs (Figure 5), which permit the formation of two zinc-binding fingers (Cole & Danos, 1987; Barbosa et al., 1989). These motifs are strictly conserved in all known E6 proteins, and their integrity appears to be essential for E6 function (Kanda et al., 1991; Sherman & Schlegel, 1996).

Identifying the localisation of E6 has been controversial and complex, partly due to its extremely low level of expression in cells. HPV-18 E6 was originally reported to be nuclear and membrane associated with a half-life of the order of 4 hours (Androphy et al., 1987a; Grossman et al., 1989). Subsequent studies also found cytoplasmic localisation of E6 (Liang et al., 1993; Chen et al., 1995), and this conflict was revisited in a recent study comparing the intracellular localisations of E6 proteins from low-risk (HPV-11) and high-risk (HPV-18) viruses (Guccione et al., 2002). This study demonstrated somewhat surprising differences in intracellular distribution of high-risk versus low-risk E6 proteins, with HPV-18 E6 showing diffuse expression throughout the cell while HPV-11 E6 was predominantly nuclear with a punctate pattern that partially colocalised with PML bodies.

E6 and apoptosis

One of the major roles of the E6 protein in the viral life cycle appears to be the abrogation of host cell apoptosis, but this can, in some cases, contribute towards the development of malignancies. Expression of either of the early viral proteins, E2 or E7 has been shown to promote apoptosis (Webster et al., 2000; Jones et al., 1997b), so it is not unreasonable that papillomaviruses have also evolved multiple strategies to counteract these pro-apoptotic effects. The first biochemical activity attributed to E6 was its ability to inactivate the p53 tumour suppressor protein (Scheffner et al., 1990). This was a fundamentally important observation since the p53 gene is frequently mutated in human cancers, and loss of the p53 surveillance function is a common feature of cancer development (Levine, 1997; Slee et al., 2004).



Cellular proteins	E6 type	Comment	References
I. Associated proteins			
E6-AP	High-risk HPVs and BPV-1	Ubiquitin ligase	Scheffner et al., 1993
E6-BP	High-risk HPVs and BPV-1	ER Ca^{++} binding protein	Chen et al., 1995
Paxillin	HPV-16 and BPV-1	Focal adhesion protein	Tong & Howley, 1997
AP-1	BPV-1	Transcriptional factor	Tong et al., 1998
Tuberin	HPV-16	Putative tumour suppressor	Elston et al., 1998
IRF-3	HPV-16	Interferon regulatory factor-3	Ronco et al., 1998
CBP/p300	HPV-16 and BPV-1	Transcriptional coactivator	Patel et al., 1999
PKN	HPV-16	Serine/threonine kinase	Gao et al., 2000
Tyk2	HPV-18	Tyrosine kinase	Li et al., 1999
Zyxin	HPV-6	Focal adhesion protein	Degenhardt & Silverstein, 2001
TNF R1	HPV-16	Tumour necrosis factor receptor 1	Filippova et al., 2002
Fibulin-1	High-risk HPVs	Extracellular matrix protein	Du et al., 2002
XRCC1	HPV-1, 8, 16	Single-stranded break repair	Iftner et al., 2002
II. Degradation targets			
P53	High-risk HPVs	Tumour supressor	Scheffner et al., 1990
Myc	HPV-16	Oncoprotein	Gross-Mesilaty et al., 1998
Bak	HPV-18 and cutaneous HPV	Proapoptotic protein	Thomas & Banks, 1998
hMCM7	Low- and high-risk HPVs	A replication licensing factor	Kuhne & Banks, 1998
E6TP1	High-risk HPVs	A putative GTPase-activating protein	Gao et al., 1999
hDlg	High-risk HPVs	Tumour suppressor	Gardiol et al., 1999
MUPP1	HPV-18	Adapter in signalling ?	Lee et al., 2000
hScrib	HPV-16	Tumour suppressor	Nakagawa & Huibregtse, 2000
Gps2/AMF-1	Low- and high-risk HPVs	Transcription factor	Degenhardt & Silverstein, 2001
MAGIs	High-risk HPVs	Tumour suppressor ?	Thomas et al., 2001, 2002
MGMT	High-risk HPVs	DNA repair protein	Srivenugopal & Ali-Osman, 2002
hADA3	High-risk HPVs	Transcriptional coactivator	Kumar et al., 2002

Figure 5. ***Sequence of the HPV-16 E6 protein.***
The sequence is arranged in a zinc-finger configuration. The amino acid residues ($_{121}$KKQR$_{124}$) and ($_{145}$TRR$_{147}$), essential for nuclear import and the PDZ domain-binding motif ($_{148}$ETQL$_{151}$) are marked. T$_{149}$ is a putative phosphorylation site for protein kinase A. On the lower panel are summarized some of the cellular binding partners of E6 (Modified from Fan & Chen, 2004).

Interestingly, in cervical cancers p53 is wild type, suggesting that the activity of E6 with respect to p53 resembles that of an inactivating mutation (Crook et al., 1991b; Scheffner et al., 1991). The p53 tumour suppressor represents a major constraint to viral replication, since, once activated by the unscheduled induction of DNA replication, it can promote cell cycle arrest or apoptosis of the infected cell (Vogelstein et al., 2000). Hence, beside high-risk HPVs, several other viruses encode proteins that functionally inactivate p53. SV40 LargeT prevents transactivation of p53 target genes through association with its DNA binding domain (Ruppert & Stillman, 1993). Ad E1B-55K abolishes the same function by binding to the transactivacting domain of p53 (Lin et al., 1994), yet in association with E4orf6 it can also lead to p53 degradation (Steegenga et al., 1998; Querido et al., 2001), while the Hepatitis B Virus X protein sequesters p53 in the cytoplasm (Elmore et al., 1997).

The mechanism by which high-risk HPV E6 proteins overcome p53 activity is well established, and involves a tripartite complex between E6, the cellular ubiquitin ligase E6-AP, and p53 (Huibregtse et al., 1993a, b). In this, E6 binds both p53 and E6-AP simultaneously and thereby acts as a bridge for targeting the ligase to p53 (Huibregtse et al., 1991), directing its ubiquitination and proteasome mediated degradation (Scheffner et al., 1993). A stretch of 18 residues in the central region of E6-AP is responsible for contacting E6, and a synthetic peptide corresponding to this region (termed the E6-AP peptide) blocks the E6/E6-AP interaction (Huibregtse et al., 1993b). E6-AP belongs to the HECT-domain family of ubiquitin ligases (Schwarz et al., 1998) and has E3 ligase activity independently of E6 (Nuber et al., 1998), nonetheless, it does not appear to be normally involved in the degradation of p53, since no association between p53 and E6-AP has been observed. Indeed, approaches aimed at blocking E6-AP activity, either by the use of antisense oligonucleotides (Beer-Romero et al., 1997) or dominant negative mutants (Talis et al., 1998), increased the levels of p53 in HPV-positive, but not in HPV-negative cells, confirming that E6-AP plays an essential role in E6 directed degradation of p53 *in vivo*, but has no effect on p53 levels in cells lacking E6. Under normal growth conditions p53 is also turned over by the ubiquitin proteasome pathway, and this is

mediated by the ubiquitin ligase Mdm2 (Honda et al., 1997), but this pathway is completely inactive in HPV-positive cancer cells, while p53 is subjected to the E6/E6-AP induced degradation entirely (Hengstermann et al., 2001; Camus et al., 2003). It is quite clear, however, that during viral infection and HPV-induced cervical lesions not all p53 is degraded, as several studies have reported detectable levels of p53 in HPV-infected cells (Mantovani & Banks, 1999; Cooper et al., 1993; Lie et al., 1999). A possible viral pathway for regulating E6 activity with respect to p53 relies on a series of polypeptides termed E6*, which are expressed by the high-risk HPV types through alternative splicing of E6 mRNA (Schneider-Gadicke et al., 1988). These alternatively spliced transcripts originate from the viral early promoter and splice out at a splice donor site about 120 bp from the initiation codon, then splice back in at, typically, four downstream splice acceptors to produce E6* I-IV mRNAs (Smotkin & Wettstein, 1986; Smotkin et al., 1989; Doorbar et al., 1990; Sherman & Alloul, 1992), as can be seen in Figure 6. For some time it was thought that this splicing event was a means of obtaining high levels of E7 expression, but additional evidence suggested that this is not the case (Stacey et al., 1995).

Although the E6* transcripts are abundant in both HPV-infected cells and in cell-lines derived from cervical tumours (Schneider-Gadicke & Schwarz, 1986; Smotkin & Wettstein, 1986), endogenous E6* protein has been detected only when HPV-18-containing cervical tumour cells were grown in nude mice (Schneider-Gadicke et al., 1988). When translated *in vitro*, E6* proteins are generally unstable (Shalley et al., 1996), suggesting that rapid turnover may explain the low levels of E6* protein observed in the cell. One of the possible functions of the E6* proteins was demonstrated by studies that showed that HPV-16 E6*IV and HPV-18 E6*I can inhibit the E6-directed degradation of p53 *in vitro* and *in vivo* (Shalley et al., 1996; Pim et al., 1997). An explanation for this activity of E6* was provided by the demonstration that HPV-18 E6*I binds both to *in vitro* translated E6-AP and to full-length E6 protein, but not to p53 itself (Pim et al., 1997). A mutational analysis of HPV-18 E6*I has shown that the antiproliferative effects of E6* overexpression appear to correlate mainly with its ability to interact with the full-length E6 protein (Pim & Banks, 1999), and to block the formation of the E6-AP/E6 E3 ligase

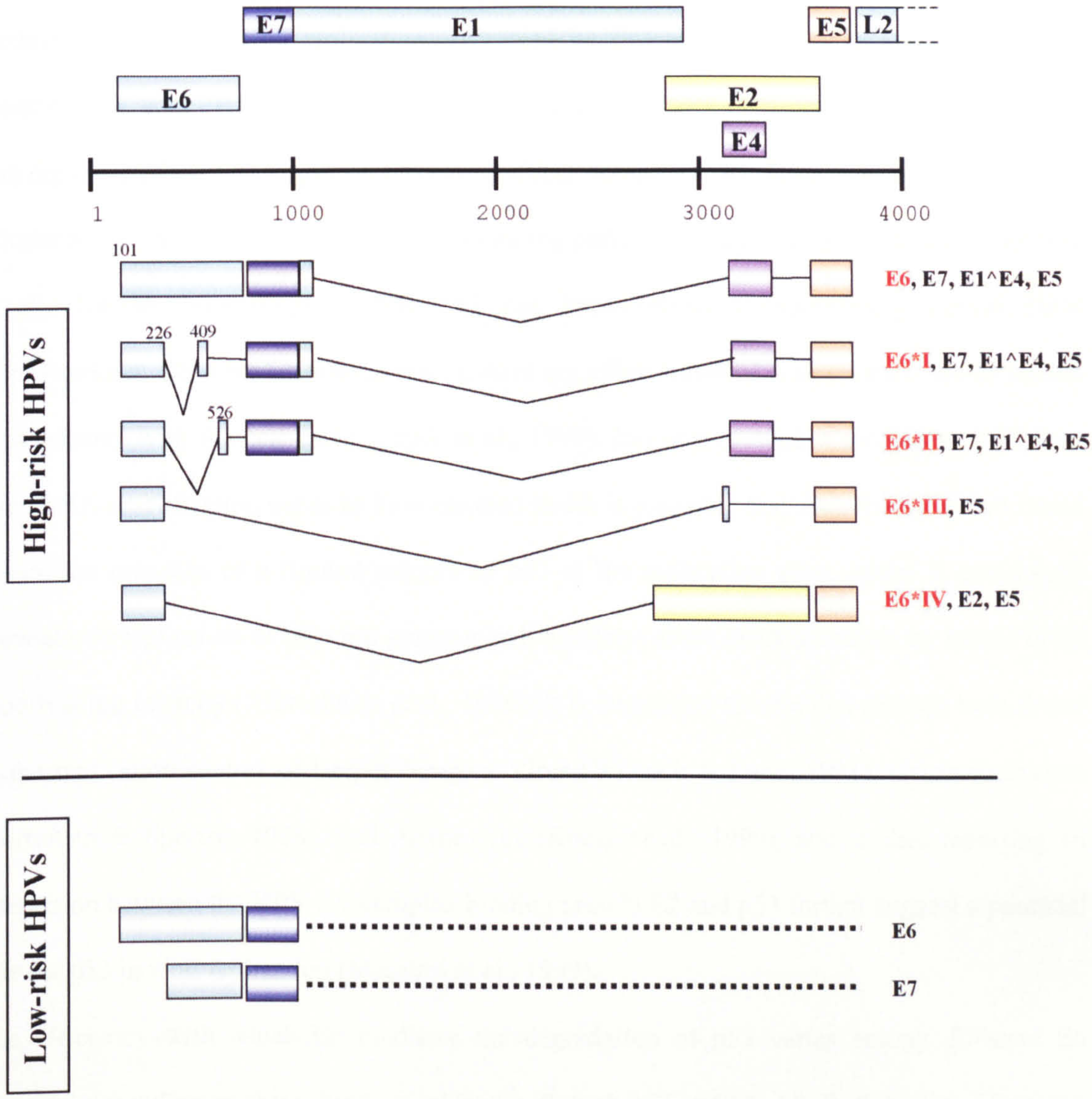


Figure 6. ***Alternative splicing in the generation of E6, E7 and E6* transcripts.***

The cartoon shows the differences between the high and low-risk mucosal virus types in transcription of the E6-E7 genes. Low-risk virus genes are transcribed in a linear fashion (lower panel) whilst alternative splicing results in at least five mRNA species in high-risk viruses (upper panel). The diagram represents the early genome region and mRNA species containing the E6 and E6 variant ORFs of HPV-16. Numbers of common splice sites are shown only once. The coding capacity of each mRNA species is indicated on the right (Modified from Shally et al., 1996; Sedman et al., 1991).

that is specific for p53. These studies suggest that one function of the E6* proteins is to modulate p53 during viral replication. Prompted by this observation, a recent study further examined the modulatory effects of E6*I on E6 and found that E6*I protein can function as a fine regulator of the full length E6 protein by direct interaction that leads both to changes in its cellular abundance as well as its distribution during particular phases of the cell cycle (Guccione et al., 2004b). Interestingly, while p53 has been shown to specifically inhibit HPV amplificational DNA replication *in vivo*, it does not affect episomal maintenance, which occurs in synchrony with the cell cycle (Lepik et al., 1998). In order to elicit a productive infection, viral DNA amplification needs to be controlled and it is plausible that the activity of E6* could ensure the presence of a limited amount of p53 at the replication sites, where it could both prevent overreplication of the viral genome and, possibly, assist DNA synthesis by means of its proofreading capacity (Albrechtsen et al., 1999). It is interesting to note that p53 has been found in the replication centres of Herpes Simplex Virus (Wilcock & Lane, 1991), Cytomegalovirus (Fortunato & Spector, 1998), and Adenovirus (Konig et al., 1999), and studies reporting an interaction between the HPV *ori*-complex binding protein E2 and p53 further suggest a potential role for p53 in viral replication (Massimi et al., 1999).

The efficiency with which E6 mediates the degradation of p53 varies among different E6 proteins, depending on their ability to interact with both p53 and E6-AP. Both high and low-risk mucosal HPV E6 proteins are able to bind the p53 C-terminus, however such interactions do not induce degradation. Binding to the core region of p53 is much greater for high-risk E6 proteins, and it is this interaction that allows efficient degradation of p53 (Li & Coffino, 1996). In addition, HPV-16 E6 binds E6-AP more strongly, and concomitantly degrades p53 more effectively, than HPV-18 E6. HPV-11 E6 has minimal levels of binding to E6-AP (Huibregtse et al., 1993b), and degrades p53 *in vivo* only weakly (Storey et al., 1998). Interestingly, the E6 proteins of both high and low-risk cutaneous HPV types do not associate with either E6-AP or p53 and are incapable of affecting p53 stability (Elbel et al., 1997), therefore the mechanism by which these viruses evade the constraints that p53 places on viral replication remain to be

determined. One possible explanation could be their interaction with the pro-apoptotic protein, Bak (Thomas & Banks, 1998, 1999). Bak is a potent inducer of apoptosis (Chittenden et al., 1995; Ink et al., 1997), and belongs to the Bcl-2 family of proteins (Reed, 1998). Bak forms pores within mitochondrial membranes resulting in a cytochrome flux and caspase induction, leading to apoptosis (Shimizu et al., 1999). During the normal processes of keratinocyte differentiation, Bak protein becomes highly expressed in the upper epithelial layers (Krajewski et al., 1996), and appears to represent a common obstacle for a broad range of HPV types that replicate in differentiating keratinocytes, since the E6 proteins of both high and low-risk mucosal HPVs, plus those of high-risk cutaneous types have been shown to inhibit Bak-induced apoptosis (Thomas & Banks, 1998, 1999; Jackson et al., 2000). As with p53, this involves targeting Bak for proteolytic degradation through a process that also involves E6-AP, probably by accelerating a normal cellular process, since Bak appears to be a substrate of E6-AP in the absence of E6 (Thomas & Banks, 1998). Degradation of Bak by HPV-11 E6 is less effective, and this correlates with a weaker anti-apoptotic activity of the low-risk mucosal HPV types (Thomas & Banks, 1999).

HPV-16 E6 has also been reported to enhance the degradation of the cellular oncoprotein, c-Myc, in an E6-AP-dependent fashion (Gross-Mesilaty et al., 1998). Unscheduled expression of c-Myc gives rise to high levels of apoptosis (Askew et al., 1991), and its down-regulation appears to be required to promote keratinocyte differentiation (Pietenpol et al., 1990a; Freytag et al., 1990). It has been demonstrated that HPV-16 E7 is able to block TGFβ-mediated downregulation of c-Myc, in order to sustain proliferation of the infected keratinocytes (Pietenpol et al, 1990b). Since, deregulated expression of c-Myc in differentiating cells induces apoptotic cell death (Askew et al., 1991), E6's degradation of c-Myc seems logical for the equilibrium between E7-promoted proliferation and cell survival.

E6 and transcription

The primary structures of the E6 proteins are poorly conserved, but all of them contain regularly

spaced Zn^{++} finger-like motifs (Cole & Danos, 1987). This structural feature suggests a possible involvement in transcriptional control. In fact, both high and low-risk E6 proteins can modulate the transcriptional activity of several cellular and viral promoters in both a p53-dependent and independent way (Sedman et al., 1991; Desaintes et al., 1992; Pim et al., 1994; Lechner et al., 1992; Lechner & Laimins, 1994; Thomas et al., 1995). However, E6 can also bind DNA, although not in a site-specific fashion (Grossman et al., 1989), suggesting that the transcriptional effects of E6 involve protein interactions, rather than E6 binding directly to promoter elements. An indication as to the mechanisms by which this may occur has come with the demonstration that low and high-risk E6s interact with p300/CBP (Patel et al., 1999; Zimmermann et al., 1999). The p300/CBP transcriptional co-activators play important roles in activating a great number of genes involved in the regulation of cell cycle, differentiation and immune response, and many viral oncoproteins have been shown to require the interaction with these co-activators for optimal transforming activity (Goodman & Smolik, 2000). HPV-16 E6 was shown to directly bind three regions of p300/CBP and this results in a downregulation of p300/CBP activation activity (Patel et al., 1999; Zimmermann et al., 1999). Both p53 and NF-κB-responsive promoters are negatively affected by this E6-mediated repression of p300/CBP (Patel et al., 1999; Zimmermann et al., 1999, Huang & McCance, 2002) and, although p300/CBP was previously shown to interact with p53, the inhibitory effect of E6 is p53 independent. NF-κB is activated upon viral infection and promotes transcription of a number of genes involved in the local immune response such as class I MHC and interleukins (Baldwin, 1996), therefore inhibition of NF-κB target genes may help the virus to disrupt the cellular antiviral response. HPV-16 E6 also binds the interferon regulatory factor 3 (IRF-3), and inhibits IFN-β induction (Ronco et al., 1998) with the same probable outcome. Finally it should also be borne in mind that the E6/p300 interaction, and its possible contribution towards survival of the transformed cell, could be a by-product of the regulation of viral gene expression. The viral E2 protein also interacts with p300/CBP (Lee et al., 2000a; Marcello et al., 2000), and this appears to involve a cellular protein, AMF-1/Gsp2, which in turn enhances p300 activity but which,

intriguingly, is also a target for E6 mediated degradation (Peng et al., 2000; Degenhardt & Silverstein, 2001). Therefore the interaction between E6 and p300 may also represent a means of downregulating E2 transcriptional activity, and thereby controlling the levels of E6 expression by a feed-back mechanism.

The ability of the high-risk E6 proteins to activate the expression of the catalytic subunit of telomerase, hTERT is another important function of E6 required for immortalisation (Klingelhutz et al., 1996; Meyerson et al., 1997; Nakamura et al., 1997). Telomerase is a multi-subunit enzyme that adds hexamer repeats to the telomeric ends of chromosomes. Telomerase activity is usually restricted to embryonic cells and is absent in somatic cells (Horikawa & Barretti, 2003). The lack of telomerase activity results in a shortening of telomeres with successive cell divisions eventually leading to senescence (Liu, 1999). In contrast, in most cancers, reactivation of hTERT expression occurs and leads to reconstitution of telomerase activity (Liu, 1999). Immortalisation of keratinocytes requires activation of hTERT and the p16 pathway (Kiyono et al., 1998; Dickson et al., 2000). At present, the precise mechanism of telomerase activation by E6 is unknown, however, it appears that E6 may transactivate the hTERT promoter through the combined action of Myc and Sp-1 (Kyo et al., 2000; Oh et al., 2001).

A role for E6 in cell-cell adhesion, cell polarity and cell proliferation

Epithelia are organized in sheets of specialized cells that are connected by various types of junctions. Such intercellular contacts are crucial for maintaining cell adhesion, cell polarity, cytoskeletal structure and for regulating cell proliferation. During carcinogenesis, loss of these characteristics leads to tissue disorganization and progression into metastasis (Hirohashi & Kanai, 2003; Martin & Jiang, 2001). Recently, studies using *Drosophila* have demonstrated that a group of tumour suppressor genes encoding membrane-associated proteins, Dlg, Scribble and Lgl, cooperate in the same pathway to regulate both epithelial structure and cell proliferation (Bilder & Perrimon, 2000; Bilder et al., 2000; Humbert et al., 2003). Two of these, Dlg and

Scribble, are multidomain proteins containing PDZ motifs, sites of protein-protein interaction involved in the clustering of ion channels, signaling enzymes and adhesion molecules for specific cytoskeletal structures found at the membranes of polarized cells (Craven & Bredt, 1998; Kim, 1997). PDZ domains typically bind to specific sequences (XT/SXV), usually found in the extreme carboxyl termini of their target proteins (Doyle et al., 1996; Songyang et al., 1997), and have been identified in a number of virus proteins that are associated with oncogenesis, including HTVL-1 Tax protein, Adenovirus 9 E4orf1 protein and the E6 proteins of high-risk HPV types (Lee et al., 1997). The fact that a number of PDZ-containing proteins have been identified as being common targets of these oncogenic virus proteins indicates that they may well belong to conserved pathways that are subverted by diverse viruses during oncogenic transformation. Indeed, all E6 proteins derived from the high-risk mucosal HPV types have a carboxy-terminal PDZ-binding domain, which is not involved in p53 binding and degradation (Pim et al., 1994; Crook et al., 1991a), but which nonetheless contributes to E6 transforming activity, since its deletion impairs E6's ability to transform rodent cells (Kiyono et al., 1997). Furthermore, transgenic mice expressing a mutant of E6 lacking the six amino acids at the carboxy terminus, $E6^{\Delta 146\text{-}151}$, demonstrate that it is necessary for E6's induction of epithelial hyperplasia *in vivo* (Nguyen et al., 2003a, b). To date, six different PDZ domain-containing proteins have been shown to be susceptible to proteasome-mediated degradation by the high-risk HPV-16 and HPV-18 E6 proteins. These include human discs large (hDlg) (Gardiol et al., 1999), MAGI-1, MAGI-2 and MAGI-3 (Glaunsinger et al., 2000; Thomas et al., 2002), MUPP1 (Lee et al., 2000b) and hScrib (Nakagawa and Huibregtse, 2000). These proteins are all characterized by having multiple protein-protein interaction motifs and are frequently expressed at sites of cell-cell contact. Of these PDZ domain-containing substrates of E6, most information is available concerning the mechanism of action of hDlg and hScrib. Based on studies in *Drosophila*, these proteins have been shown to act in concert to regulate the processes of cell growth and cell attachment (Woods et al., 1996; Bilder & Perrimon, 2000; Bilder et al., 2000). In higher eukaryotic cells less is known about their function, although Dlg has been

reported to induce growth arrest in mouse fibroblasts (Ishidate et al., 2000) and a Dlg truncation mutant result in impaired morphogenesis and perinatal death during murine development (Caruana & Bernstein, 2001). Human hDlg colocalises with E-cadherin at adherens junctions of epithelial cells (Reuver & Garner, 1998; Ide et al., 1999), and interacts through different domains with several proteins, including Shaker-type K^+ channels (Kim et al., 1995), cytoskeletal protein 4.1 (Lue et al., 1994; Marfatia et al., 1996), and the APC tumour suppressor protein (Matsumine et al., 1996). hScrib protein, expressed at epithelial tight junctions, is the human homologue of *Drosophila* tumour suppressor Scribble, which cooperates with Dlg and Lgl to control both formation of cell junctions and inhibition of epithelial cell growth, possibly through controlling the localisation of growth factor receptors and signaling molecules (Bilder & Perrimon, 2000; Bilder et al., 2000). Interestingly, E6 was sufficient to disrupt tight junctions in mammalian cells, which is consistent with results from *Drosophila*, where apical junctions are disrupted in *scrib* or *dlg* mutants (Humbert et al., 2003). This suggests that degradation of hScrib and hDlg proteins by E6 proteins may be important for the loss of polarity seen in the development of HPV-mediated cervical carcinomas (Nakagawa and Huibregtse, 2000). Consistent with this notion, the increased binding affinity of hDlg to HPV-18 E6 compared with HPV-16 E6 correlates with increased hDlg degradation, enhanced transformation ability and the increased malignancy of HPV-18 (Gardiol et al., 1999; Pim et al., 2000; Watson et al., 2002). Together these data suggests that hDlg and hScrib may be important targets in the late stages of HPV induced cervical carcinomas (Mantovani & Banks, 2001). Much less is known about the activity of the other PDZ domain-containing substrates of E6. MAGI-1 has been shown to interact with β-catenin (Dobrosotkaya & James, 2000), while MAGI-2 and MAGI-3 have been shown to be involved in the downregulation of PKB activity through an interaction with the PTEN tumour suppressor (Wu et al., 2000a, b). These findings suggest that all three MAGI family members may be involved in the regulation of cell proliferation. In the case of MUPP1, a large multi-PDZ domain-containing scaffolding protein with a putative role in signal transduction (Becamel et al., 2001), there is, as yet, no clear information on its potential

biological activities. Importantly, a recent study provided strong evidence that at least three of E6's PDZ domain-containing substrates, hDlg, MAGI-1 and MUPP1, have potent tumour suppressor activities (Massimi et al., 2004).

It has recently been shown that E6 can bridge the interaction between hScrib and the E6-AP ubiquitin ligase, which normally would not recognize hScrib (Nakagawa and Huibregtse, 2000). This is reminiscent of p53 degradation, yet a clear role for E6-AP in hScrib degradation *in vivo* has not been confirmed. In contrast, proteasome degradation of hDlg appears to be independent of E6-AP (Pim et al., 2000, 2002). Furthermore, the mechanisms by which E6 degrades other targets, MAGI proteins and MUPP1 are still unknown, invoking the possible involvement of other ubiquitin ligases, identification of which is now an intensive area of research.

E6 and α-helix cellular partners

Several E6-binding partners contain similar amino acid motifs in their E6 binding domains (Chen et al., 1998; Elston et al., 1998; VandePol et al., 1998). Sequence comparison revealed a consensus sequence, LxxϕLsh, where L indicates the conserved leucine residues, ϕ is a hydrophobic residue (usually leucine), h is an amino acid residue with a side-chain capable of accepting hydrogen bonds (Asp, Glu, Asn or Gln), s represents a small amino acid residue (Gly or Ala), and xx is a dipeptide where one of the residues is Asp, Glu, Asn, or Gln (Be et al., 2001). Mutation of the conserved hydrophobic residues in the motif eliminates binding, whereas mutation of most other residues significantly reduces binding (Be et al., 2001; Chen et al., 1998; Bohl et al., 2000). Previous work demonstrated that peptides containing these consensus sequences clearly have a tendency to form an α-helix (Be et al., 2001; Chen et al., 1998; Hoellerer et al., 2003). The E6-binding partners that associate with E6 via this motif, are referred to as α-helix partners (Nguyen et al., 2002) (Figure 7). One such α-helix partner is E6-AP, the cellular ubiquitin ligase necessary for E6-mediated degradation of p53. Other α-helix partners include E6BP (Chen et al., 1995), a calcium-binding protein of the CREC family; paxillin (Tong & Howley, 1997; Tong et al., 1997; VandePol et al., 1998), a focal adhesion

protein; E6-TP1 (Gao et al., 1999), a putative Rap1 GAP protein; Bak (Thomas & Banks, 1998, 1999), a proapoptotic protein; and IRF3 (Ronco et al., 1998), a transcriptional regulator involved in interferon response. The hMCM7 (Kuhne & Banks, 1999), a component of the DNA replication licensing complex, has a perfect L2G box motif (Kuhne & Banks, 1998), however, it has a lysine residue in the place of the first conserved leucine residue, which makes its definition as α-helix partner questionable. Binding of α-helix partners to E6 has been found to be important for the transforming activity of the E6 protein of BPV-1 (Bohl et al., 2000; Tong et al., 1997). However, the role of interactions with α-helix partners in the transformation activities of HPV E6 is controversial, since E6 mutants unable to bind and degrade E6TP1 failed to immortalize HMEC (Gao et al., 2001), but E6 mutants impaired in binding to E6-AP and E6BP were still able to do so (Liu et al., 1999). A recent study has shown that a mutant of HPV-16 E6, $E6^{I128T}$, was deficient in binding α-helical partners in the skin of transgenic animals, but still able to induce epithelial hyperplasia, albeit to a lesser extent than wild-type E6 (Nguyen et al., 2002). Thus, although α-helix partner binding and p53 inactivation are involved in the induction of hyperproliferation, they are not solely responsible for it.

E6 as a therapeutic target

Since HPV E6 is one of the two major oncoproteins encoded by the virus, and is invariably expressed together with the E7 oncoprotein in cervical lesions and responsible for their malignant progression, this protein represents an attractive candidate for therapeutic intervention in HPV-induced disease. In addition to the well-documented role of E6 in transformation, it is also essential component of the productive viral infection, since both high and low-risk HPVs deleted in E6 are completely defective for initiating the viral life cycle in primary keratinocytes (Thomas et al., 1999a; Oh et al., 2004b), suggesting that E6 also represents a good therapeutic target for treating low-risk HPV infection. Numerous approaches have been directed against the polycistronic E6/E7 mRNA to block the expression of both E6 and E7 in HPV-positive cervical cancer cells. This includes selectively inhibiting viral

Consensus E6 binding sequence:		**L**xxϕ**L**sh
E6-AP	(402-418)	PESSELT**L**QEL**L**GEERR
E6TP1	(1736-1752)	LASKVDQ**L**EGM**L**KMLRE
E6BP	(201-217)	NGDGFVS**L**EEF**L**GDYRW
Paxillin	(327-343)	PPKPGSQ**L**DSM**L**GSLQS
IRF3	(133-149)	SDTQEDI**L**DEL**L**GNMVL
Bak	(189-205)	LNVLVV**L**GVV**L**LGQFVV
Mcm7	(634-650)	IRLMEMSKDSL**L**GDKGQ

Figure 7. ***Sequence alignment of several E6-binding partners*** (α-helix partners), which contain similar amino acid motifs in their E6 binding domains. This seven amino acid motif, which form part of an α-helical structure that is necessary for the interaction with E6 is underlined and the conserved leucines (L) are shown in red (Modified from Kuhne & Banks, 1999).

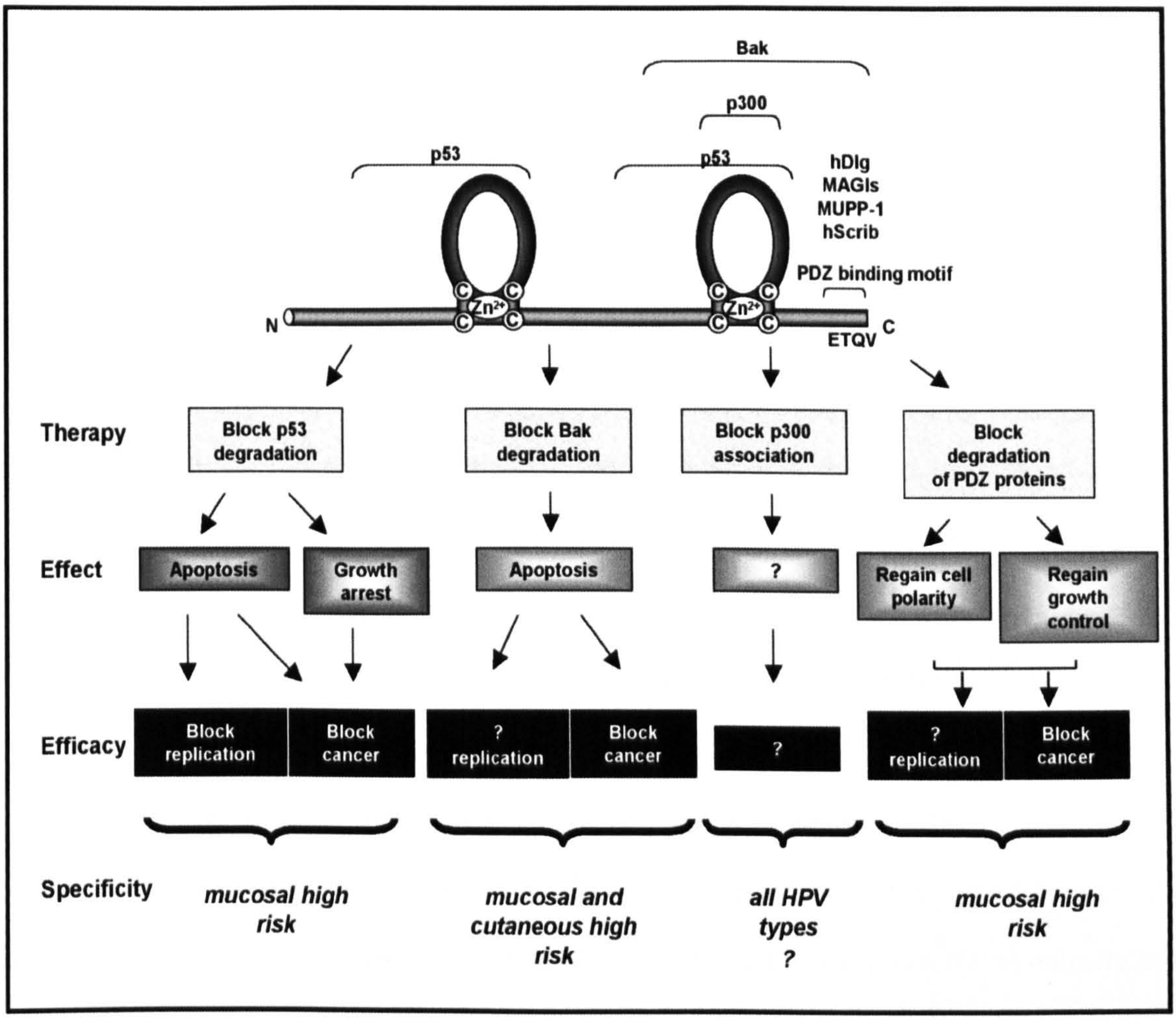


Figure 8. ***Schematic diagram of the E6 protein showing the regions involved in interactions with some of its target cellular proteins that offer the most potential as targets for chemotherapeutic intervention.*** Also shown are the potential consequences of the therapy upon the normal viral life cycle as well as on cancer development for different HPV types.

transcription (Goodwin & DiMaio, 2000), or by using antisense constructs (Hamada et al., 1996; von Knebel Doeberitz et al., 1992), ribozymes (Alvarez-Salas et al., 1998), or short-interfering RNA (siRNA) (Hall & Alexander, 2003; Butz et al., 2003). All of these approaches cause growth suppression or apoptotic cell death, demonstrating the feasibility of reactivating functional tumour suppressor pathways in HPV-positive cells. However, since a major role of E6 appears to be the abrogation of host cell apoptosis, it can be reasoned that blocking the activity of E6, while retaining that of E7 as a proapoptotic stimulus, might provide a higher therapeutic potential. This would be difficult to obtain by targeting the viral mRNAs, since the E6 and E7 genes are expressed together as polycistronic transcripts, but it could be accomplished by using peptides to selectively block the E6 protein functions. Indeed, administration of peptide aptamers was found to induce apoptosis of HPV-positive cancer cells (Butz et al., 2000). Thus blocking the E6-mediated degradation of p53 is a major therapeutic goal, since there is strong evidence that the p53-responsive pathways are fully functional in cervical tumour cell lines (Butz et al., 1995, 1999), and reactivation of p53 would then bring about growth arrest and/or apoptosis of the HPV transformed cells. However, this is unlikely to be universally applicable, since inhibition of E6-induced degradation does not always lead to increased p53 levels. In several cervical cancer cell lines p53 can be stabilised only after additional genotoxic insult, indicating a lack of intrinsic activation of p53 despite the presence of the viral oncogenes (Mantovani & Banks, 1999; Finzer et al., 2002). It has been shown that interactions between viral proteins and PDZ domain-containing proteins constitute a general mechanism for virus-induced oncogenesis, hence this class of interactions might represent ideal therapeutic targets for the later stages of virus-induced disease. Because the structures of a number of PDZ domains have been solved (Morais et al., 1996; Doyle et al., 1996; Hillier et al., 1999), and the binding motif of E6 is small and exposed, the rational design of relatively nontoxic chemotherapeutic agents, capable of specifically inhibiting the interaction between E6 and this class of targets should be possible (Figure 8). Although the high-risk HPV E6-p53 and E6-PDZ interactions are likely to be excellent therapeutic targets in both normal viral infection

and in virally induced cancer, it is not clear whether they will be as relevant for other HPV types. Low-risk mucosal HPV E6 proteins can bind p53, but their effect on p53 steady state levels is minimal. In addition, their lack of a PDZ binding domain suggests that PDZ proteins are not relevant for the normal life cycle of low-risk HPV types. The same is also true for cutaneous types, where inactivation of p53 does not appear to be a conserved feature of these viruses and, again, they also lack PDZ binding sequences. Two protein targets of these other viral E6 proteins that are worth considering as potential points for therapeutic intervention are the proapoptotic protein Bak and the transcriptional co-activator p300/CBP (Figure 8). In the case of p300, this would appear to be a common target of both low and high-risk HPV, plus BPV-1 E6 proteins, indicating an evolutionarily highly conserved function of E6 (Patel et al., 1999; Zimmermann et al., 1999, 2000). In addition, in the case of high-risk mucosal E6 proteins, this activity would also seem to play a role in its ability to transform cells (Bernat et al., 2002). The interaction between HPV E6 and Bak provides an alternative target for therapeutic intervention. Bak is targeted for degradation by E6 proteins derived from both the high-risk cutaneous and the low and high-risk mucosal types (Thomas & Banks, 1998, 1999; Jackson et al., 2000). Unfortunately, the precise details of the association and the biological consequences of the E6/p300 and E6/Bak interactions are still poorly defined, making thoughts of rational therapeutic design somewhat problematic. Therefore, although E6 interactions with both p300 and Bak have great potential in the future, significantly more work is required before they can be considered as serious drug discovery candidates.

Activities of the E7 protein

The E7 proteins from all the HPV types are small, highly conserved, multifunctional proteins of approximately 100 amino acids (Munger et al., 2001). They can be divided into three conserved domains: CD1, CD2, and CD3 (Figure 9). CD1 and CD2 share homology with conserved regions 1 and 2 (CR1 and CR2) of Adenovirus E1a, and CD2 also shares homology with SV40 large T antigen. The CD3 domain of E7 consists of a cysteine loop structure in the C-terminal

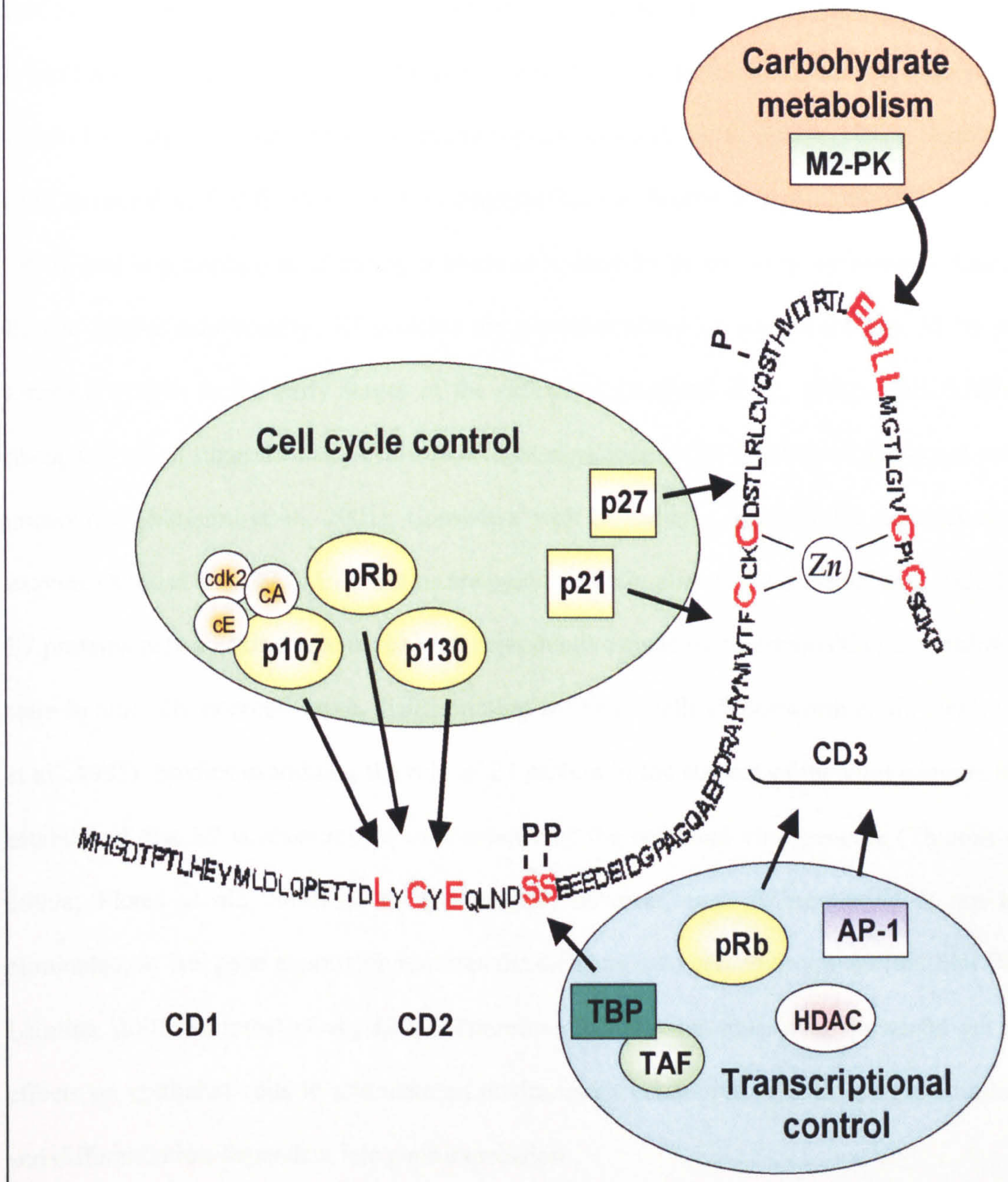


Figure 9. ***Physical interaction of HPV-16 E7 with cellular target proteins.***

The primary amino acid sequence of the HPV-16 E7 protein is given and the zinc-finger structure at its C-terminus is included in the model. Some of the cellular proteins that are known to bind E7 are grouped according to their putative interaction site on E7 and their function in the cell (Modified from Zwerschke & Jansen-Durr, 2000).

half of the protein that has been shown to bind zinc (Barbosa et al., 1989; McIntyre et al., 1993). It has been speculated that the similarity between this cysteine loop and the two cysteine loops within E6 suggest evolution from a common predecessor (Cole & Danos, 1987). Furthermore, the C-terminal half of E7 is required for oligomerization (McIntyre et al., 1993; Clemens et al., 1995), and is phosphorylated during S phase at residue 71 by an unknown kinase (Massimi & Banks, 2000). Additionally, E7 proteins are phosphorylated on serines 31 and 32 by casein kinase II (CKII) in the early stages of the cell cycle (Barbosa et al., 1990). This differential phosphorylation suggests a cell-cycle dependent regulation of E7 function which is not yet well understood (Massimi et al., 2001). Consistent with E7 being a substrate for the nuclear CKII enzyme (Krek et al., 1992), E7 proteins are predominantly nuclear (Smith-McCune et al., 1999). E7 proteins play a critical role in the viral reproductive cycle by re-establishing the proliferative state in normally nonreplicative, differentiating epithelial cells (Woodworth et al., 1992; Cheng et al., 1995). Studies examining the role of E7 protein in the context of the viral genome further established that E7 is essential for maintenance of the episomal viral genome (Thomas et al., 1999a; Flores et al., 2000). It is also critical, however, that differentiation is not totally eliminated, as late gene expression requires the differentiated cell environment (del Mar Pena & Laimins, 2001; Hummel et al., 1995). Therefore, E7 proteins must exert powerful yet subtle effects on epithelial cells to stimulate an environment conducive to both episomal replication and differentiation-dependent late gene expression.

The first activity of E7 to be described was its association with members of the retinoblastoma (pRb) tumour suppressor family to facilitate progression into S phase. The pRb proteins play an important role in cell cycle regulation, promoting the transition from G1 into S phase. In normal cells, pRb is unphosphorylated in early G1 and becomes increasingly phosphorylated towards S phase. The unphosphorylated forms of pRb form complexes with the E2F/DP1 transcription factors and actively repress transcription from promoters containing E2F sites (Dyson, 1998). A large number of genes required for DNA synthesis, such as DNA polymerase alpha and thymidine kinase, are transcribed in a cell cycle-dependent manner because of regulation by

E2F (La Thangue, 1994; Slansky & Farnham, 1996). E7 binds to the B-pocket domain of pRb through the LXCXE motif, resulting in the release of free E2F, which then upregulates a variety of genes involved in cell-cycle control and DNA synthesis (Munger et al., 2001; Zwerschke & Jansen-Durr, 2000). In normal epithelia, cell cycle exit occurs following differentiation and is believed to be mediated in a large part by the action of the pRb protein (Liu et al., 2004). By binding to pRb, E7 promotes cell cycle progression in differentiated epithelial cells, allowing the productive replication of HPV genomes. Additionally, the E7 ubiquitin-mediated degradation of pRb appears to be essential to efficiently overcome cell cycle arrest, since transformation deficient cutaneous HPV-1 E7 also binds pRb efficiently, but is unable to destabilize pRb (Giarre et al., 2001; Gonzalez et al., 2001), showing that the E7-mediated degradation of pRb correlates strongly with the ability of E7 to induce cell transformation. Besides pRb, E7 interacts with two other members of the same family, p107 and p130, which also negatively regulate E2F transcription (Dyson et al., 1989; Davies et al., 1993). As mentioned above, binding to the pocket proteins by E7 results in their degradation through the proteasome pathway (Boyer et al., 1996). The exact mechanism of pRb destabilisation, however, remains unclear, and although E7 interacts with the S4 subunit of the 26S proteasome (Berezutskaya & Bagchi, 1997), this does not seem the correct link, since a mutant lacking this interaction domain can still efficiently degrade the pocket protein (Gonzalez et al., 2001). The E7 protein itself is a target of ubiquitin-dependent proteolysis (Reinstein et al., 2000) and a recent study has proposed that the SCF-Skp2 ubiquitin ligase complex causes ubiquitination of E7, which is consequently degraded in specific nuclear bodies (Oh et al., 2004a). Whether this complex is also recruited by E7 to degrade the pRb protein remains to be determined.

In addition to binding to pRb family members, E7 proteins associate with cyclins A and E as well as with cyclin-dependent kinase (cdk) inhibitors p21 and p27 (Davies et al., 1993; Funk et al., 1997; Jones et al., 1997a; Tommasino et al., 1993; Zerfass-Thome et al., 1996). High-risk E7 proteins bind cyclinA-cdk2 complexes directly, and HPV-18 E7 also binds cyclin E indirectly through p107 (McIntyre et al., 1996). In both cases, binding to E7 retains cdk2-

associated kinase activity (McIntyre et al., 1996; Ruesch & Laimins, 1998). The high-risk E7s also act to increase the levels of the cyclin A and E proteins, while low-risk E7 proteins have no such effect (Martin et al., 1998). Two cdk inhibitors, p27 and p21, have also been shown to bind to E7, which presumably blocks their action and thus further enhances the activities of the cdks (Funk et al., 1997; Jones et al., 1997a; Zerfass-Thome et al., 1996). Consistent with this notion, it was shown that inactivation of both the pRb protein and p21 by E7 is necessary to prevent cell cycle arrest (Helt et al., 2002).

Besides overriding the cell cycle controls, mainly at the G1/S boundary (Zwerschke & Jansen-Durr, 2000), which appears to be essential for the ability of E7 to reactivate S phase-promoting genes in quiescent host cells, E7 modulates the expression of additional cellular genes that are not directly involved in cell cycle control by binding to AP-1 transcription factors, including c-Jun, JunB, JunD and c-Fos (Antinore et al., 1996). It has been suggested that through interactions with AP-1 factors and pRb HPV E7 can modulate both the process of cell cycle progression as well as cell differentiation. On one hand, by binding to c-Jun independently of pRb, E7 may potentiate the activation of genes involved in early cell cycle progression and thus promote S phase entry. On the other hand, by associating with both pRb and c-Jun, E7 may de-regulate keratinocyte differentiation via disruption of pRb/c-Jun complexes. As such, the differential targeting of AP-1 factors and pRb provides a potential mechanism by which E7 could uncouple differentiation from cell cycle progression. E7 has been also found to associate with members of the basal transcriptional machinery, the TATA-binding protein (TBP) and the TBP-associated factor-110 (TAF-110) (Massimi et al., 1997; Phillips & Vousden, 1997; Mazzarelli et al., 1995). The HDACs play an important role in cell cycle regulation, and are consequently potential targets for viral oncoproteins. In fact, the CD3 zink finger domain of E7 has been found to interact with HDAC-1 (Brehm 1998; Brehm et al., 1999) and this provides yet another method by which E7 can de-repress gene transcription, and may explain the essential nature of the CD3 domain in activating E2F-regulated genes as well as immortalizing keratinocytes.

Late proteins

The viral late functions, such as vegetative viral DNA synthesis, capsid protein synthesis, and virion assembly occur exclusively in differentiated keratinocytes (Doorbar, 1998). Viral and cellular events which influence HPV late gene expression are only poorly understood because until recently there has been no convenient system for mimicking productive infection *in vitro* (Stanley, 2001). Studies on naturally occurring warts have revealed the virus to encode three late proteins: L1 and L2, which are virion coat proteins (Doorbar & Gallimore, 1987), and E4, a nonstructural late protein (Doorbar et al., 1986).

E4 protein

The coding region for E4 lies in the early portion of the viral genome overlapping the E2 ORF, yet the E4 protein is heavily expressed late in infection (Doorbar et al., 1986; Frattini et al., 1997; Rotenberg et al., 1989). Typically, E4 proteins are expressed from spliced RNAs that encode an E1^E4 fusion protein with the start codon and 5 amino acid residues provided by the E1 portion (Nasseri et al., 1987), but it will be referred to here simply as E4. E4 proteins are present in the more superficial layers of differentiating mucosal epithelium (Palefsky et al., 1991), often in coordination with viral genome amplification (Doorbar et al., 1997) and they are thought to be involved in vegetative viral DNA replication (Davy et al., 2002; Roberts et al., 2003), since E4-deficient viruses cannot complete the viral life cycle and vegetative genome amplification is abolished (Peh et al., 2004). Whilst it is the most abundantly expressed of the HPV encoded proteins, E4 protein is poorly conserved and varies greatly in predicted size and sequence (Doorbar et al., 1989). However, even with this tremendous diversity of E4 proteins, a common function is suggested by the retention of several common properties including a high proline content, general hydrophilicity, oligomerisation (Ashmole et al., 1998; Bryan et al., 1998), and the presence of short conserved sequence motifs (Roberts et al., 1997).

The first function ascribed to the E4 proteins from the high-risk types was the ability to collapse the cytokeratin matrix which, it is reasoned, could contribute to virion release (Doorbar et al.,

1991). It has further been shown that overexpression of HPV-11 and HPV-16 E4 induces a G2 arrest in a variety of cell types (Davy et al., 2002). Based on these activities it is thought that the cytoskeleton could act as a scaffold to which E4 can attach host cell proteins, thereby allowing their function to be modified, or preventing them from translocating to appropriate subcellular compartments. In agreement with this, activated cyclin B/cdk1 is found in the cytoplasm together with E4 and keratins, and this is believed to be the mechanism by which E4 arrests cells in G2 (Davy et al., 2002; Wang et al., 2004). This may then provide a suitable environment for viral genome amplification. High-risk HPV E4 proteins may also play a role in regulating gene expression, since they interact with an RNA helicase, E4-DBD, which is a member of a helicase family involved in mRNA splicing and transport as well as in initiation of translation (Doorbar et al., 2000). Two other recent observations of novel E4 properties are worthy of mention. First HPV-16 E4 was shown to associate with mitochondria leading to apoptosis (Raj et al., 2004). And the second observation was that HPV-1 E4 induced a reorganisation of PML bodies (Roberts et al., 2003), which in conjunction with the known functions of L2 (see below) strongly implicates papillomaviruses as targeting these nuclear structures in a fashion analogous to other DNA viruses (Barber, 2001).

L1 and L2 capsid proteins

L1 and L2 are the classic late viral proteins that are expressed after viral genome replication and which co-assemble to form a 55-60 nm virion capsid structure (Howley & Lowy, 2001). L1, a 55 kDa protein, is the major component of the capsid; L2 is slightly larger at 70 kDa, but is only a minor constituent of the virion. Capsid formation is an intrinsinc property of L1, and so-called viral-like particles (VLPs) will spontaneous form from L1 expressed in the absence of other viral proteins (Banks et al., 1987a; Kirnbauer et al., 1992; Zhou et al., 1992; Hagensee et al., 1993). These VLPs are antigenically equivalent to authentic virions (Sapp et al., 1996) and form the basis for an effective prophylactic vaccine (Koutsky et al., 2002). However, incorporation of L2 is required for the effective encapsidation of viral DNA (Roden et al., 1996; Zhou et al.,

1994).

How natural virions form within infected cells has been a subject of much interest in recent years. A common theme emerging from multiple studies is that L2 plays an important role in regulating virion assembly. L2 accumulation in the host cell nucleus precedes that of L1, and L2 localises to PML bodies (Florin et al., 2002a). PML bodies are poorly understood nuclear entities composed of the PML protein and numerous affiliated proteins (Borden, 2002; Bernardi & Pandolfi, 2003; Dellaire & Bazett-Jones, 2004). Many functions have been ascribed to PML bodies, including anti-tumour and anti-viral roles, and disruption of PML bodies is observed following infection by several different DNA viruses (Regad & Chelbi-Alix, 2001; Salomoni & Pandolfi, 2002). The association of L2 with PML bodies also appears to cause some reorganisation as there is release of at least one PML component, Sp100 (Florin et al., 2002b). Similar studies with BPV-1 also found that L2 was necessary for localisation of L1 to the PML bodies (Day et al., 1998) and additionally seemed to recruit E2 as well (Day et al., 1998; Heino et al., 2000; Okoye et al., 2005). Localisation of L2, L1, and E2 in the PML bodies may simply provide a convenient means of concentrating viral factors to facilitate virion assembly. Alternatively, even subtle disruption of PML bodies may have consequences for viral replication and persistence that have not yet been evaluated. The recent finding that L2 interacts with five additional host proteins, PATZ, PLINP, PMSP, tubular-nephritis antigen related protein (TIN-Ag-RP), and the chaperone Hsc70, further suggests a more complex role for L2 than simply an inert capsid structural protein (Gornemann et al., 2002, Florin et al., 2004).

Objectives of the study

From the above introduction it is clear that the HPV life cycle is linked to the differentiation program of the infected keratinocyte and is tightly regulated by a number of viral proteins. The E6 and E7 gene products subvert the cell growth-regulatory pathways and modify the cellular environment to facilitate viral replication in a cell that is terminally differentiating and has exited the cell cycle. At the same time, E1 and E2 act to regulate viral gene expression and viral DNA replication. Finally, the late viral gene products are expressed; E4 taught to facilitate viral egress, as well as L1 and L2, the two viral capsid proteins. In addition to the known specific functions of these viral proteins, there are several examples of regulatory interactions between different viral gene products. These include E1-E2 (Liu and Melendy, 2002), E2-L2 (Heino et al., 2000; Okoye te al., 2005), and L1-L2 (Xi & Banks, 1991). These observations prompted us to search for new possible interactions between viral proteins. In this study, we have observed, and performed the first extensive analysis, of the interaction between E6 with E2 and E6 with L2 proteins. The biological meaning and the relevance of these interactions will be discussed in detail.

Another important feature which is clear from the above discussion is that HPVs are important human pathogens. Prevention or elimination of these infections would not only benefit the numerous patients with benign lesions, but ultimately should reduce the incidence of cervical cancer and possibly other epithelial cancers as well. Unfortunately, progress in vaccine development has been slow and no specific anti-papillomavirus agents are available. Development of molecular strategies aimed at disrupting the functions of the viral proteins to eliminate infections thus seems to be the way of choice. Hence, we describe in the second part of the thesis the use of small synthetic peptides that bind to the E6 oncoprotein to block its ability to degrade certain cellular proteins. The molecular mechanisms by which E6 targets its cellular substrates for degradation will also be studied using these peptides.

Results and Discussion

PART 1: *Cross-talk between HPV proteins*

HPV encoded proteins frequently have multiple roles in the virus life cycle and can co-operate with each other to achieve their effect. For example, several functions have been attributed to E2. In the basal layer of the epithelium, E2 plays a role in genome maintenance (Penrose & McBride, 2000), whereas in the upper epithelial layers, the protein is required for genome amplification. A key role for E2 in viral DNA replication is to bind the viral E1 DNA helicase and to target it to the origin of DNA replication (Lowy & Howley, 2001). However, in the upper layers of the epithelium, E2 is also thought to improve the efficiency of genome packaging into capsids by direct binding to the capsid protein L2, as well as to the viral DNA (Day et al., 1998). Together, this suggests that the function of E2 is different at different stages of the viral life cycle, and that these different functions of E2 may in part depend on the background of HPV encoded proteins with which it is coexpressed. These considerations prompted us to search for other possible interactions between different HPV proteins since, whilst great emphasis has been placed on identifying cellular targets of the viral proteins, little attention has been given to potential interplay between the viral proteins themselves.

In order to investigate potential interactions between different HPV proteins, a series of *in vitro* GST pulldown assays were performed. The HPV-16 E1, E2, E4, E6, E7, and L1 proteins were expressed in *E. coli* as GST-fusion proteins, bound to glutathione resin, and assayed for their ability to associate with *in vitro* translated HPV-16 E2, E6, E7 or L2 protein. Each GST-fusion protein was incubated with one of the *in vitro* translated radiolabelled HPV proteins as indicated in Figure 10 for 1 hr at 4°C. Complexed proteins were then washed three times in PBS containing 0.5% NP40, and bound proteins were monitored by SDS-PAGE and autoradiography. The gels of the binding assays were also stained with Coomassie blue to

confirm equal loading of the GST-fusion proteins (Figure 10, lower panels). The interactions between E1 and E2 (Mohr et al., 1990; Ustav & Stenlund, 1991), E2 and L2 (Day et al., 1998; Heino et al., 2000; Okoye et al., 2005), and L1 and L2 (Xi & Banks, 1991) have been described previously and served as positive controls for these pulldown experiments. As can be seen from Figure 10, there is no association between E6 and E7 (Figure 10, panel c), nor do they associate with E1, E4 or L1 (Figure 10, panels a, d and e, respectively). Surprisingly however, E2 and L2 not only interact with each other, as shown previously (Day et al., 1998; Heino et al., 2000; Okoye et al., 2005), but also with all the HPV proteins tested in this assay: E1, E4, E6, E7, and L1 (Figure 10, panels a-e). These assays were repeated at least three times and were quantified by scanning with a PhosphorImager in order to quantify the percentage of input protein binding in the assays, and the collated results are shown in Table 1. It can clearly be seen that L2 binds strongly to all the HPV proteins tested with more than 30% of load retained. The same was also observed for the interactions between E2 and E1, E4 and L1 proteins. In contrast the E2-E6 interaction is somewhat less strong with approximately 25% of the load being retained. Interestingly, there were significant differences in the interaction between the E2 and E7 proteins, depending on which of the two proteins was used as a GST-fusion protein. The E2-E7 interaction is very weak when E2 is used as a GST-fusion protein, with approximately 8% of the input being bound (Figure 10, panel b). However, this increases dramatically when GST-E7 is used, with approximately 34% of the input of E2 being retained (Table 1 and Figure 10, panel c), suggesting that there are either posttranslational modifications or that the E7 binding site on E2 is not exposed when E2 is fused to GST. Taken together, these *in vitro* binding data reveal potential new interactions between the following combinations of HPV encoded proteins: E1-L2, E2-E4, E2-E6, E2-E7, E2-L1, L2-E4, L2-E6, and L2-E7. An intriguing question arising from these data is whether E2 and L2 therefore represent key players in the regulation of the viral life cycle through interactions with other virally encoded proteins. To investigate this in more detail, we focused our attention on two of these potential interactions: E2 with E6 and L2 with E6, since the HPV encoded E6 proteins have multiple activities that have been implicated

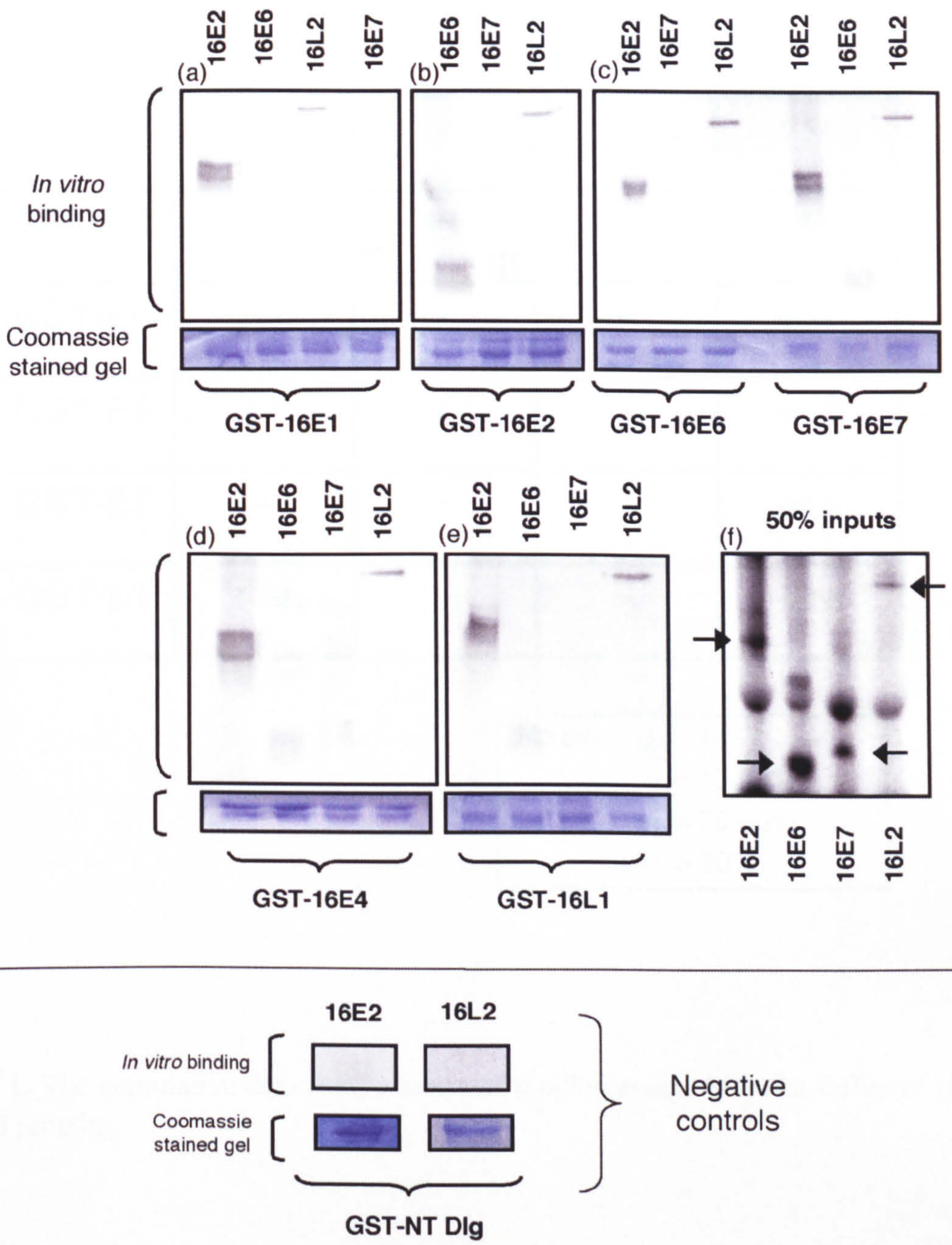


Figure 10. Cross-talk between HPV encoded proteins.
Radiolabelled *in vitro* translated HPV-16 E2, HPV-16 E6, HPV-16 E7 and HPV-16 L2 proteins were incubated with purified GST-16 E1 (a), GST- 16 E2 (b), GST-16 E6 (c), GST-16 E7 (c), GST-16 E4 (d) and GST-16 L1 (e), as indicated, for 1 hour at 4°C. Following extensive washings bound proteins were detected by SDS-PAGE and autoradiography. After exposure, the gels were rehydrated and stained with Coomassie brilliant blue to show GST-fusion protein inputs (lower part of each panel). Panel (f) shows 50% of the *in vitro* translated protein inputs: the arrows on the left of the Figure mark the positions of the *in vitro* translated E2 and E6, the arrows on the right indicate the L2 and E7 proteins. Note that the N-terminal portion of a cellular protein, NT-Dlg was used as a negative control. Assays were repeated at least three times and equivalent results were obtained.

	E2	E6	E7	L2
GST-E1	+++	-	-	+++
GST-E2	/	++	-	+++
GST-E4	+++	-	-	+++
GST-E6	++	/	-	+++
GST-E7	+++	-	/	+++
GST-L1	+++	-	-	+++

No binding < 10%
+ = 10-20%
++ = 20-30%
+++ > 30%

Table 1. The cumulative data from a series of binding assays between different HPV encoded proteins.

in the development of cervical cancer.

E2-E6

Whilst numerous studies have assigned different activities to the HPV E2 and E6 proteins, most of these analyses have been performed using only individual viral proteins, without the other viral gene products that would be encountered in the context of a viral infection. Furthermore, there are a number of reports showing that the overexpression of the E2 protein in HPV-positive cancer cell lines, including SiHa, Caski, and HeLa cells, which contain integrated viral DNA and actively express E6/E7, induces G1 growth arrest or apoptosis (Hwang et al., 1993; Dowhanick et al., 1995; Francis et al., 2000; Goodwin et al., 2000; Desaintes et al., 1997; Webster et al., 2000; Demeret et al., 2003). Because of this, and based on the potential association between E2 and E6, we initiated a series of studies to investigate the potential biological and biochemical consequences of E2 and E6 coexpression.

HPV E2 induces relocalisation of the HPV E6 oncoprotein

Previous studies had reported a predominantly nuclear localisation for the HPV-16 E2 protein (Day et al., 1998), and more diffused pattern of expression for the high-risk HPV E6 proteins (Sherman & Schlegel, 1996; Guccione et al., 2002). To investigate the effects of E2 and E6 upon each other we first analysed the cellular localisation of E2 and E6, either alone or in combination. U2OS cells were transfected with plasmids containing HPV-16 E2 and HA-tagged HPV-18 or HA-tagged HPV-16 E6. After 24 hrs the cells were fixed and stained with rabbit polyclonal anti-E2 and mouse anti-HA antibodies. The results obtained are shown in Figure 11 (panels A and B) and, as can be seen, HPV-18 E6 alone shows a diffused pattern of expression in agreement with previously published data (Guccione et al., 2002 and Figure 11A, panels a and i). However, when E2 is cotransfected, we observed a significant accumulation of E6 within the nucleus (Figure 11A, panel c-e). Similarly, HPV-16 E6 shows very strong nuclear localisation in the presence of E2 (Figure 11B). This pattern of expression was never seen when

E6 was expressed alone, suggesting that E2 induces the redistribution of the E6 protein into the nucleus. In order to confirm that this activity of E2 was not cell type specific, we repeated the assay in the immortalized keratinocyte HaCaT cells and, as can be seen from Figure 11C, similar results were also obtained.

Having observed a change in the cellular localisation of high-risk HPV E6 proteins when coexpressed with E2, we next wanted to assess whether the same was true for the low-risk HPV types. In this study we performed cotransfection experiments in U2OS cells with the HA-tagged HPV-11 E6 protein and GFP-tagged HPV-11 E2. The results are shown in Figure 11D. In contrast to the results obtained with high-risk proteins, the low-risk HPV-11 E6 and E2 proteins are localised mainly in the nucleus and exhibit a marked punctate pattern of expression (Figure 11D, panels a and b, respectively). This is consistent with previous reports showing that both HPV-11 E6 and HPV-11 E2 colocalise with the PML protein, the main component of PML oncogenic domains (PODs) (Swindle et al., 1999; Guccione et al., 2002). When we coexpressed the two proteins, we observed two different patterns of expressions. In a subset of cells positive for both HPV-11 E2 and HPV-11 E6 no colocalisation was observed, although the two proteins showed a punctate pattern of expression (Figure 11D, panels c-e, left side cell). However, we also observed a subset of cells, where the two proteins colocalised in dot like structures (Figure 11D, panels c-e, right side cell). Taken together these data suggest that the status of the cell cycle may determine whether low-risk HPV-11 E2 and E6 colocalise.

HPV E2 and HPV E6 proteins concentrate within SC-35 domains

Upon closer examination of cells coexpressing the high-risk HPV E2 and E6 proteins it is clear that their expression is not uniformly distributed through the nucleus, but rather that there are clear areas of concentration of both proteins (Figure 11A, panels f-h). To investigate whether these areas correspond to known nuclear structures, we performed cotransfection experiments in U2OS cells with HA tagged HPV-18 E6 and HPV-16 E2 proteins. Due to the unavailability of a triple staining system, one of the proteins could not be visualised, however, since we knew from

A.

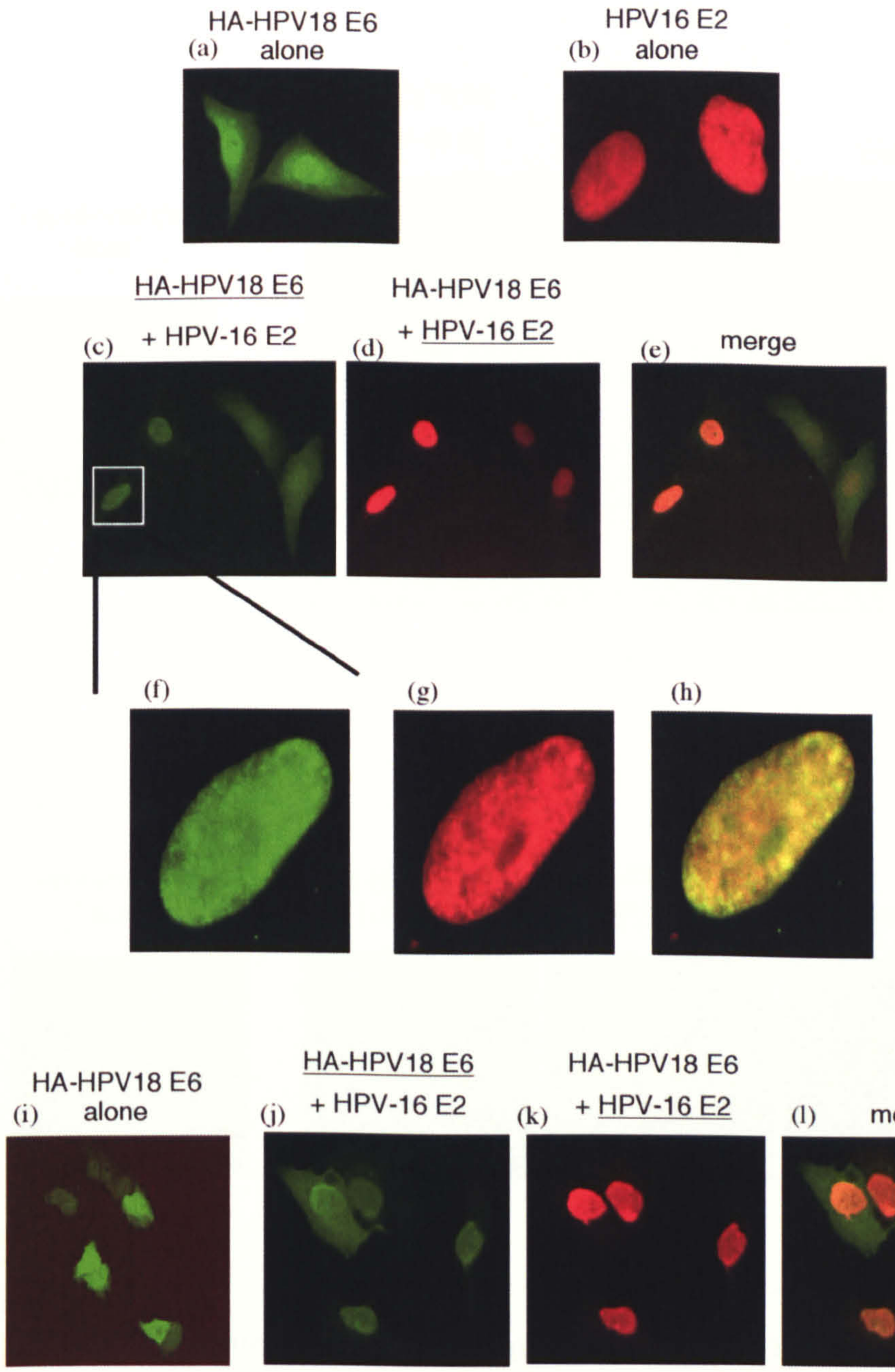
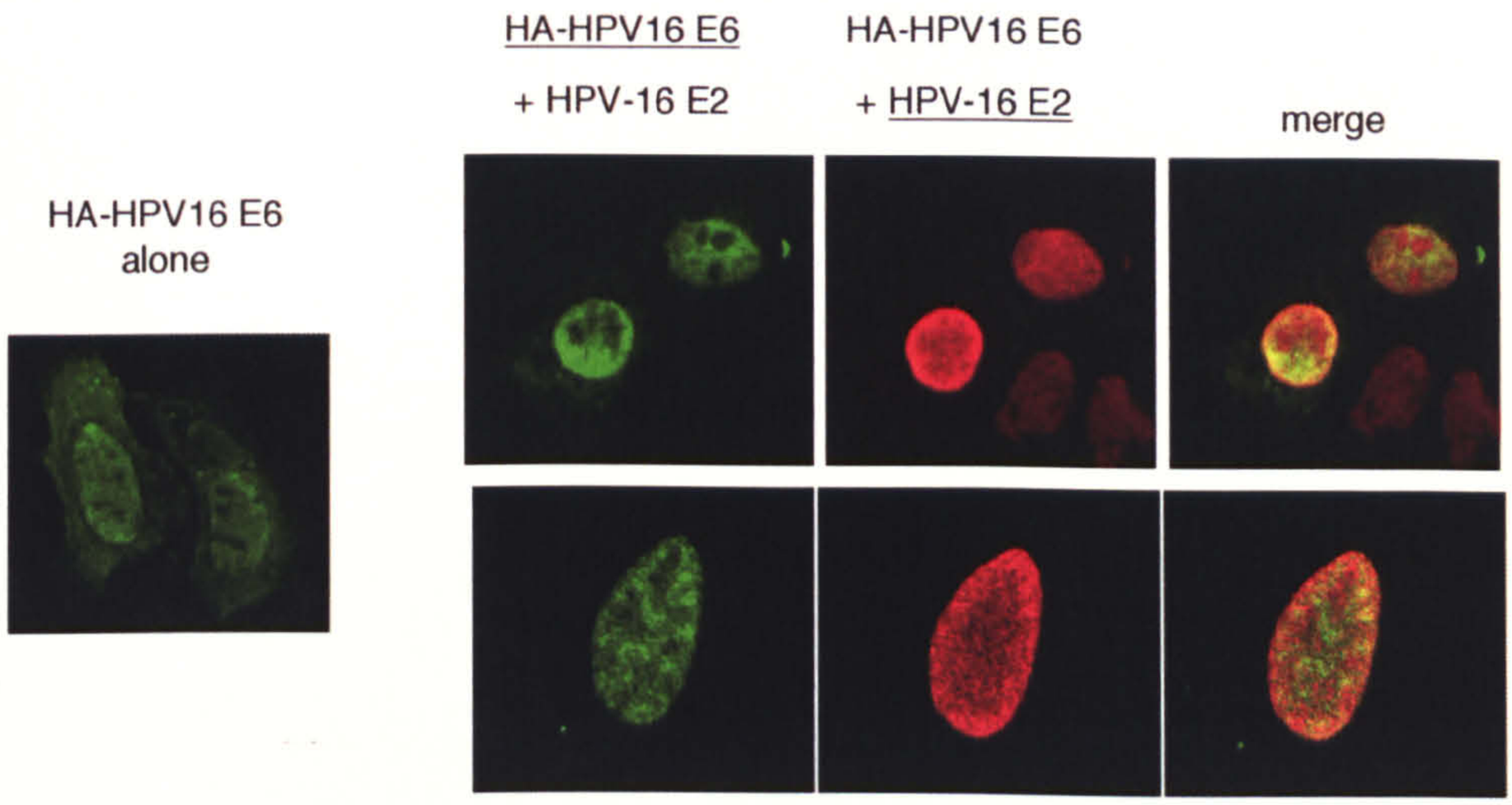


Figure 11. HPV E2 induces relocalisation of the HPV E6 oncoprotein.
(A) E2 expression affects the subcellular distribution of HPV-18 E6. U2OS cells were either transfected with HA-18 E6 alone (panels a and i) or CMV-16E2 alone (panel b) or in combination (panels c-e, j-l and insets f-h) and then probed by double immunofluorescence. The E2 protein was detected with the anti-E2 antiserum and rhodamine-conjugated goat anti-rabbit antibody (Molecular Probes). HA-18 E6 was detected with the anti-HA monoclonal antibody, 12CA5 (Roche) and FITC-conjugated goat anti-mouse IgG. Note increased nuclear retention of E6 in the presence of E2 (panels c and j) as well as speckled nuclear staining visible upon higher magnification (inset c expanded to f-h). In each experiment a total of 10 fields were counted, and in this case out of 380 cells showing positive staining for both E2 and E6, 57 showed nuclear retention of E6. Similar percentages were obtained in each of three replicate experiments.

B.



C.

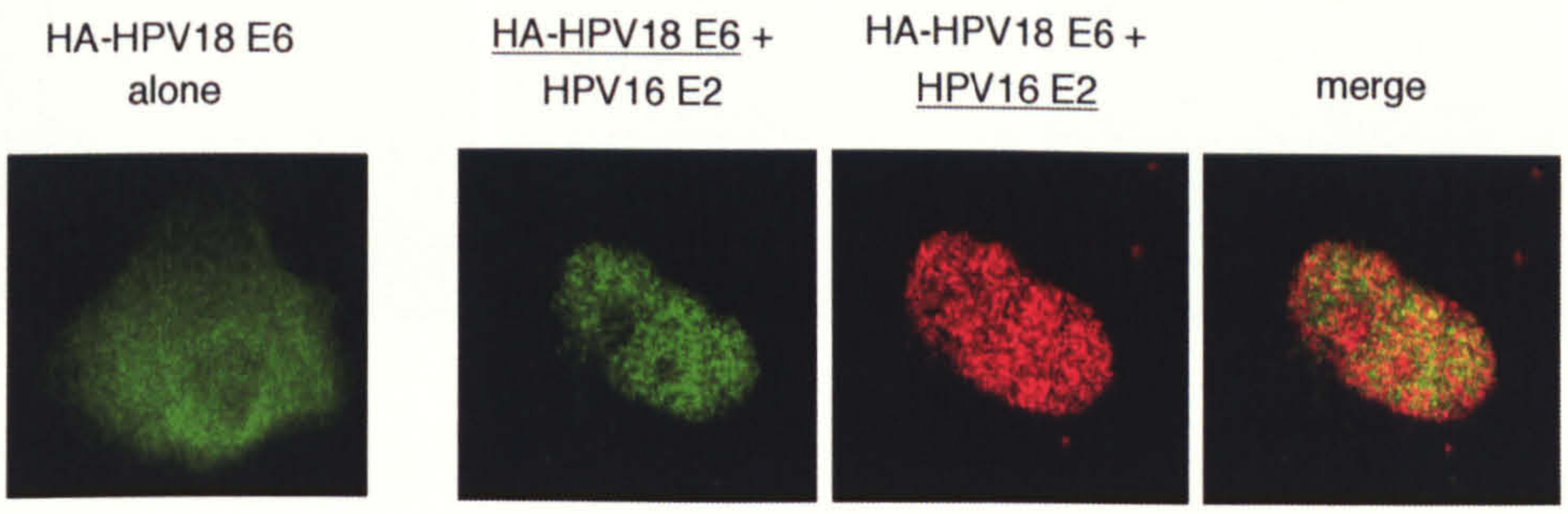


Figure 11. HPV E2 induces relocalisation of the HPV E6 oncoprotein.
(B) E2 expression affects the subcellular distribution of HPV-16 E6. U2OS cells were either transfected with HA-16 E6 alone or in combination with HPV-16 E2 and then detected by double immunofluorescence (indicated by underlining) as described in 11A. In each experiment a total of 20 fields were counted, and in this case the E6 showed exclusively nuclear staining in the presence of E2 in 29 cells of 223 cells analysed. Similar percentages were obtained in each of three replicate experiments. (C) E2 expression affects the subcellular distribution of HPV-18 E6 also in HaCaT cells. The transfections were performed as described in 11A, and were double-stained for HA-E6 and E2 proteins (indicated by underlining). In each experiment a total of 30 fields were counted and the same proportion of cells as in panel A and B was showing E6 nuclear retention in the presence of E2. Similar percentages were obtained in each of three replicate experiments.

D.

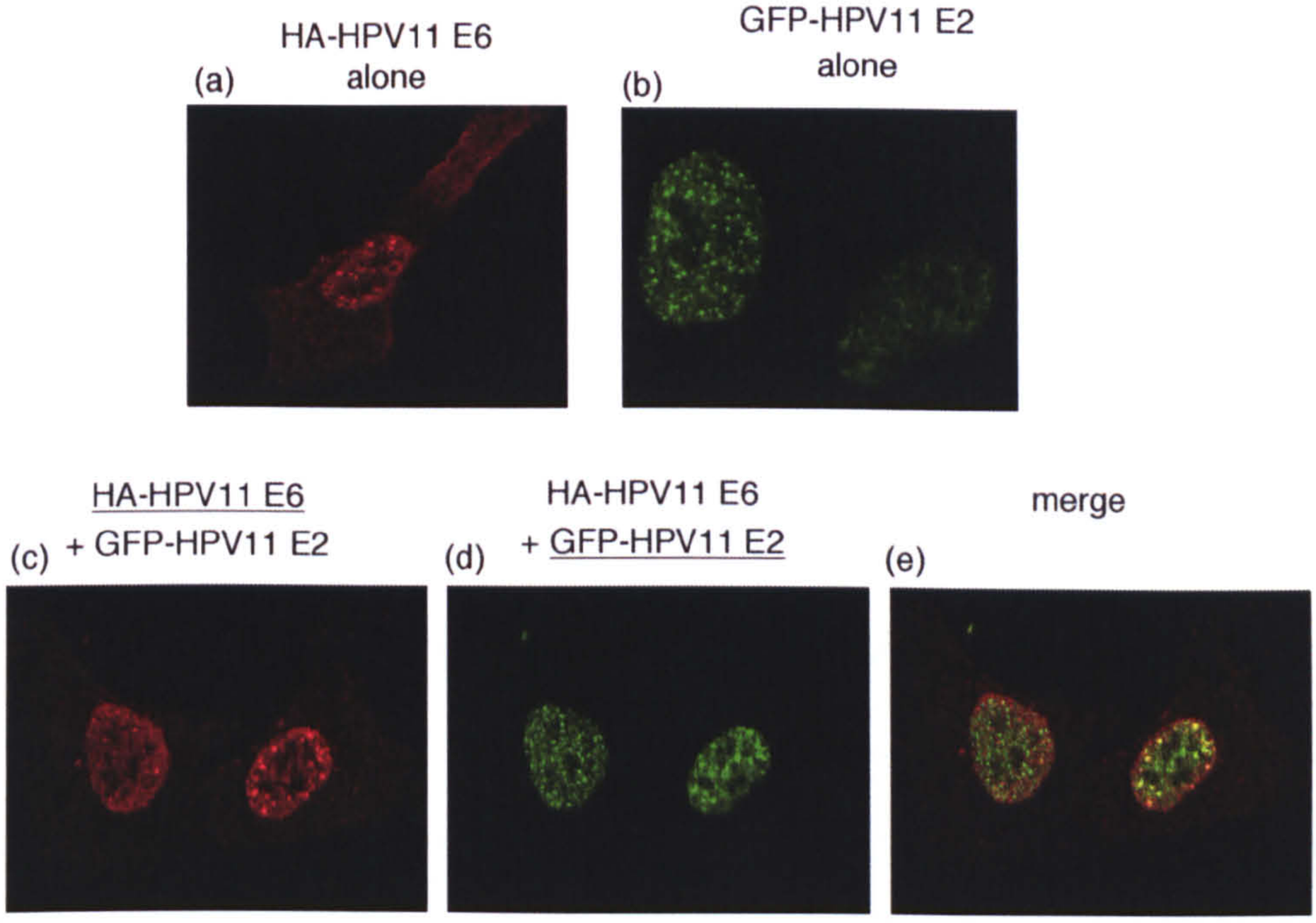


Figure 11. HPV E2 induces relocalisation of the HPV E6 oncoprotein.
(D) Localisation of HA-tagged HPV-11 E6 (a) and GFP-tagged HPV-11 E2 (b) alone or in combination (c-e) in transiently transfected U2OS cells. Cells were fixed in PBS/3.7% PFA, permeabilised with PBS/0.1% Triton X-100 and stained for anti-HA (red, 12CA5, Roche).

the previously obtained data (Figure 11A) that cells showing strong nuclear localisation of E6 also express E2, we performed coimmunostaining with an HA-specific antibody to detect E6 and a number of known nuclear proteins. As seen in Figure 12 (panels a-c), we found no colocalisation of the E6/E2 complexes with the PML protein that localises in subnuclear organelles called PODs (Borden, 2002). However, when we compared the distribution of E6/E2 complexes with that of SC-35 protein staining, we observed a nearly complete overlap in protein distribution (Figure 12, panels j-l). The SC-35 is a spliceosome assembly factor that localises in subnuclear organelles known as splicing factor compartments (SFCs) (Dundr and Misteli, 2001; Moen et al., 1995; Stein et al., 2000). The SC-35 distribution appeared to be unaffected by the coexpression of E6/E2 and neither E2 nor E6 alone colocalised with the SC-35 (Figure 12, panels d-f and g-i, respectively).

HPV E2 is recruited into PODs by HPV E6 non splicing mutant (NS)

The high-risk but not the low-risk HPVs express, through alternative splicing of the E6 mRNA, a series of polypeptides termed E6* (Schneider-Gadicke & Schwarz, 1986). In a recently published study the subcellular localisation of the full length HPV-18 E6 and one of its alternatively spliced products, E6*I, was assessed (Guccione et al., 2004b). The construct expressing a non-splicing full-length HPV-18 E6 mutated at the splice donor site (E6 NS) showed a punctate pattern of expression, being largely confined to the nucleus in discrete dots, shown to be confocal with PML (Guccione et al., 2004b and Figure 13, panel a). We therefore proceeded to investigate the distribution of E2 and E6 in the context of a mutated E6 gene no longer capable of expressing E6*. Cells were again transfected with the HA-tagged HPV-18 E6 NS mutant together with the E2 expression plasmid and the results obtained are shown in Figure 13. As can be seen, no change in the speckled staining pattern of E6 NS was observed after coexpression of E2. In contrast, however, E2 assumed the punctate staining pattern of the E6 NS mutant in the cells that coexpressed the two proteins (Figure 13, panels b-d). Although the staining pattern seen with the anti-SC-35 antibody was similar to that seen with the E6 NS

mutant, it was evident from the merged image that these regions were exclusive (Figure 13, panels e-g and h-j). Based on these observations we can conclude that both E2 and E6 can radically affect each other's pattern of expression when present within the same cell. Thus, in the context of wild type E6, expressing both full length E6 and E6*I, E2 stimulates E6 accumulation within the nucleus in domains confocal with SC-35. In contrast, in the context of a mutant E6 no longer capable of encoding E6*I, E6 itself accumulates in PODs to which it also recruits E2.

E2-mediated relocalisation of high-risk E6 is cell cycle regulated

As can be seen from Figure 11A (panels c-e and j-l), only a subset of E2-expressing cells show changes in E6 localisation, therefore we wanted to determine the percentage of these cells. Confocal microscopic analysis of transfected U2OS cells (n=200) revealed that, of 100% cells positive for E2 and E6 staining, 12% showed strong nuclear accumulation of the E6 protein. Differences in patterns of expression in immunofluorescence assays can often be explained by differences in the phase of the cell cycle. To test this hypothesis we performed immunostaining for BrdU, a marker for DNA synthesis and hence for cells in S phase. Asynchronous cultures of U2OS cells, cotransfected with E6 and E2, were pulse-labeled with BrdU for 1 hr and BrdU incorporation was analysed by immunostaining with BrdU-specific antibody. Since we previously observed a specific pattern of expression of E6 protein in the presence of E2 (Figure 11A), we stained only for E6 and looked for cells showing complete nuclear localisation. The results are shown in Figure 14 and, as can be seen, E6 shows nuclear staining both in S-phase cells (positive BrdU staining) (Figure 14, upper panel) and in cells not in S-phase (negative for BrdU) (Figure 14, lower panel). The pattern of BrdU staining through S phase has been well documented. During early S phase hundreds of small foci are distributed throughout the nucleoplasm with the exception of the nucleoli. During mid S phase replication foci concentrate around the nucleoli and in the nuclear periphery. In late S phase replication foci decrease in number but increase in size, and often take on characteristic ring and horseshoe-like structures

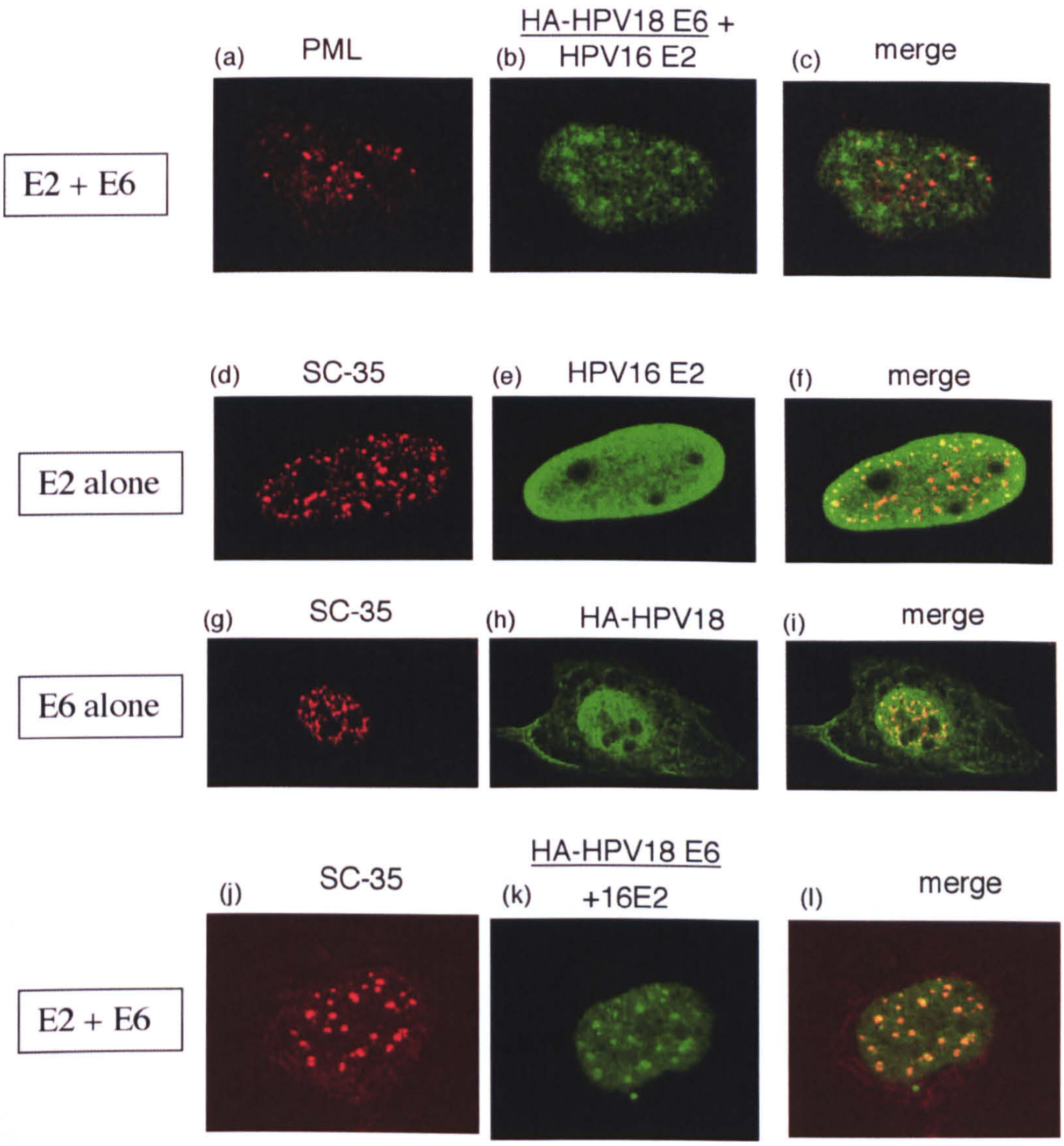


Figure 12. ***E2 and E6 in complex colocalise with the SC-35 protein.*** Transfections with constructs expressing E2 alone, HA-E6 alone or in combination were performed in U2OS cells. After 24 hours cells were fixed and double-stained for E2 using anti-E2 polyclonal antibody, for HA-E6 using an anti-HA rabbit polyclonal antibody (Y-11, Santa Cruz) and either for endogenous SC-35 using an anti-SC-35 monoclonal antibody (S4045, Sigma) or for endogenous PML using an anti-PML monoclonal antibody (PG-M3, Santa Cruz). Panels a-c show double staining for E6 and endogenous PML when E2 was cotransfected, panels d-f double staining for E2 and endogenous SC-35, panels g-i for E6 and endogenous SC-35, and panels j-l for E6 and endogenous SC-35 when E2 was cotransfected. Note colocalisation of E6 with SC-35 only in the presence of E2 (panels j-l). In each experiment a total of 15 fields were counted, and in this case out of 400 cells showing positive staining for both E2 and E6, 52 showed colocalisation of E6 with SC-35 in the presence of E2. Similar percentages were obtained in each of three replicate experiments.

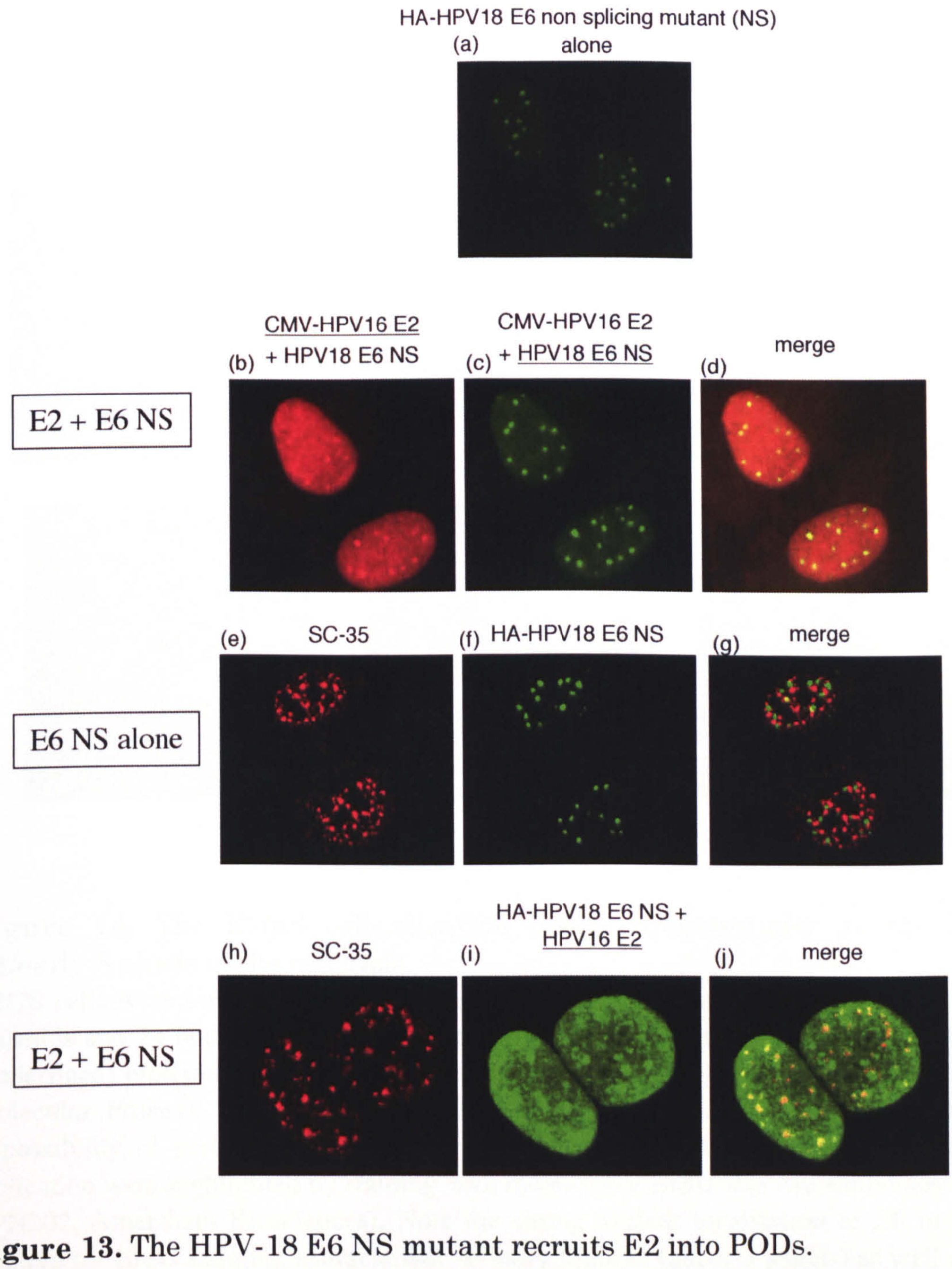


Figure 13. The HPV-18 E6 NS mutant recruits E2 into PODs.
U2OS cells were transfected with a construct expressing HA-tagged HPV-18 E6 NS alone, which expresses only full length E6 protein and which accumulates in PODs (panel a) and in combination with HPV-16 E2. E6, E2 and SC-35 were detected as for Figures 11A and 12. Note colocalisation of E2 and E6 NS in nuclear dots (panels b-d), these are not confocal with SC-35, neither when E6 NS is expressed alone (panels e-g) nor when E6 NS is expressed in combination with E2 (panels h-j). In three separate experiments a total of 10 fields were counted, and each time colocalisation of E2 and E6 NS in nuclear dots was observed.

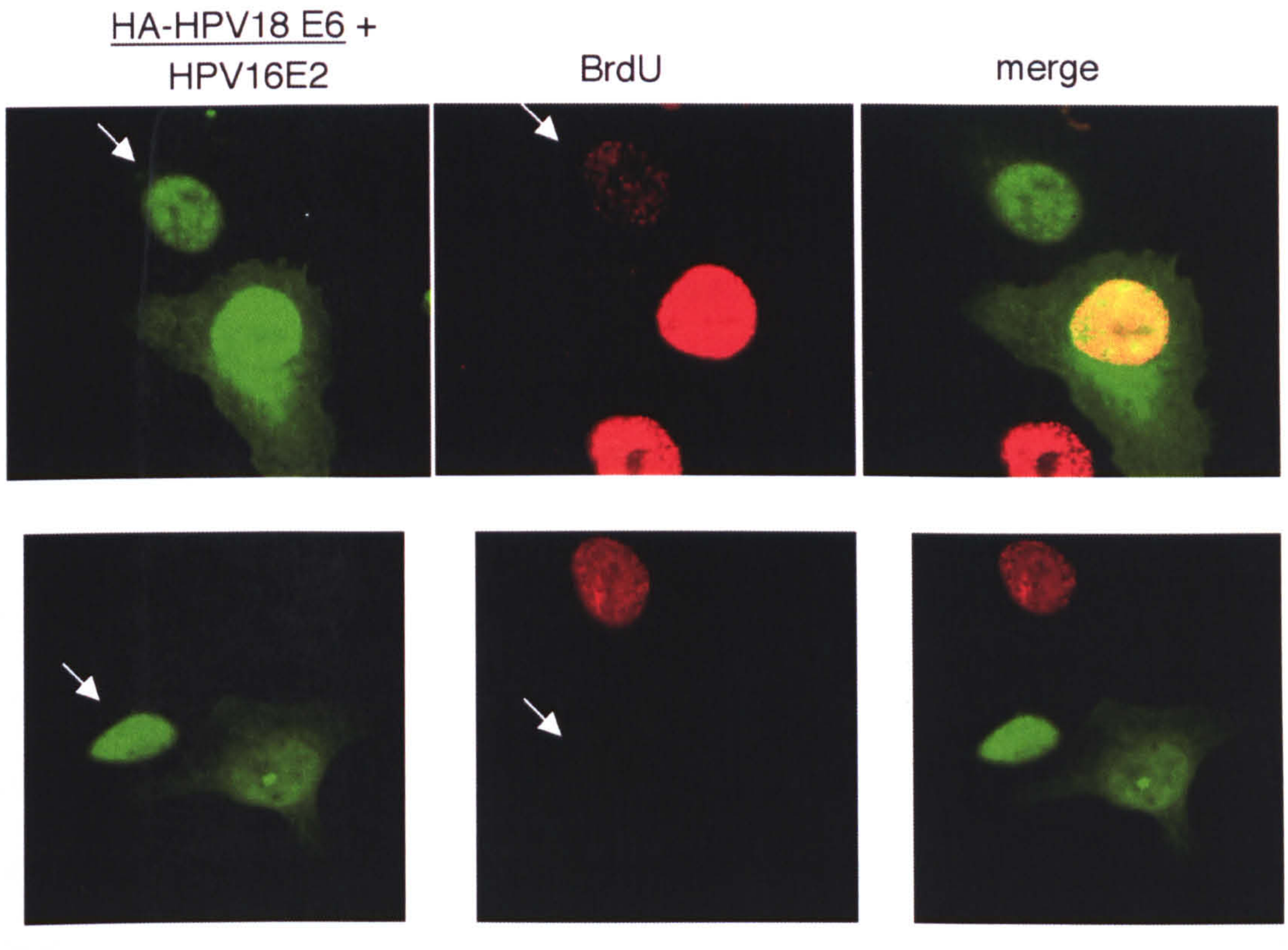


Figure 14. The E2/E6 colocalisation occurs preferentially at the late G1/early S phase of the cell cycle.

U2OS cells were transfected with HA-tagged HPV-18 E6 and HPV-16 E2 expression plasmids and 24 hours later pulse-labeled with BrdU, fixed and stained for HA-18 E6 (underlined) protein (primary: 12CA5, Roche; secondary: fluorescein-green anti mouse, Molecular Probes). The coexpressed E2 is not detectable in this case, due to the impossibility of performing a triple staining. The subnuclear sites of ongoing DNA replication were highlighted by staining with monoclonal BrdU-specific antibodies (red, RPN202, Amersham Biosciences). Note the strong nuclear localisation of E6 in cells positive for BrdU staining, characteristic of early S phase (upper 3 panels) as well as in cells negative for BrdU staining (lower 3 panels). Out of 1000 cells analysed in 5 different assays, an average of 12-15% of cells showed nuclear retention of E6 in the presence of E2. 50% of those were positive for BrdU staining.

(Leonhardt et al., 2000). The BrdU staining in cells with E6 nuclear accumulation (Figure 14, upper panel) resembles that described for early S phase, suggesting that at this phase of the cell cycle the E2/E6 functional complex forms. In addition, we also observed cells negative for BrdU staining, but positive for E6 nuclear staining, raising the possibility that the complex is formed also at other phases of the cell cycle.

*HPV16 E2 upregulates the levels of HPV-18 full length E6 and E6*I*

Having shown that E2 and E6 coexpression alters their cellular localisation, we were next interested in investigating whether they had any effects on their respective levels of expression. To do this, U2OS cells were transfected with HPV E6 and E2, either alone or in combination, and after 24 hrs cells were harvested and divided into nuclear/cytoplasmic soluble fractions and the nuclear insoluble fraction. The expression levels of E2, E6 and E6* in the different cellular fractions were then determined by western blot analysis. The results obtained are shown in Figure 15A. As can be seen, E2 expression results in a slight increase in the levels of full length E6 protein expression in the soluble fraction, but the increase in the levels of E6 in the insoluble fraction is dramatic. In contrast, the E6*I splice product shows a dramatic increase in the levels of expression in the soluble fraction and only a slight increase in the insoluble fraction in the presence of E2. Interestingly, the level of E2 expression does not change in the soluble fraction, whereas there is a marked increase in the E2 levels in the presence of E6 in the insoluble fraction (Figure 15A). To exclude the possibility that these observations merely reflect differences at the mRNA level, U2OS cells were transfected with E6 and E2 as before, together with a GFP expression plasmid as an internal control. Total RNA was extracted and subjected to RT-PCR with primers specific for full length E6 and the E6*I spliced product. As shown in Figure 15B, no change in the E6 splicing pattern was observed in the presence of E2, suggesting that the differences in the levels and patterns of protein expression are all manifested at the protein level. Taken together, this results suggests that E2 stabilises both full length E6 and its alternatively spliced form E6*I.

E2 binds to the E6 protein directly

A major aim of many previous studies has been to attempt to define the underlying reasons why the high-risk HPV types are transforming, whereas the low-risk HPV types are not. Some of the differences are clearly linked to their respective abilities to interact with their cellular targets. However, there is very little information on whether the high- and low-risk virally encoded proteins "cross-talk" in the same way. We were therefore interested to determine whether there were any differences between the oncogenic and non-oncogenic HPV E6 proteins with respect to their interaction with the E2 protein. To do this, bacterially expressed GST-16 E2 fusion protein was immobilized on glutathione-agarose and then mixed with *in vitro* translated ^{35}S-labeled HPV-16 or 18 E6 proteins. GST and GST-Dlg were also included in the assay as negative and positive controls, respectively. As can be seen from Figure 16, HPV-16 E6 and HPV-18 E6 proteins were specifically retained on the E2-conjugated agarose beads, indicating that both high-risk E6 proteins can interact with E2. Interestingly, when the assay was performed with low-risk HPV 11 E6 and HPV 11 E2, no interaction was observed (Figure 16). Taken together, these data suggest that the ability of E2 and E6 to interact is specific to high-risk HPV types. Since this pulldown assay uses *in vitro* translated proteins, there was always the possibility that the binding observed could have taken place via an intermediate protein. Therefore, to determine whether the E2 and E6 proteins can interact directly, we performed a far western blot overlay assay. Purified GST alone or BSA (negative controls), GST-E6 and GST-E1 (positive control) were immobilized on nitrocellulose. This was then overlaid with purified soluble E2 expressed as a His-tagged fusion protein (His-E2) and, following extensive washing, bound E2 was detected by western blot. The results obtained are shown in Figure 17A and reveal strong binding of E2 to GST-E6 and GST-E1 and no detectable binding to the GST alone or to BSA. In the reverse experiment, equal amounts of purified His-E2 were immobilized on nitrocellulose, and the overlay was carried out with purified GST-16 E6. Bound proteins were visualised by probing the membrane for GST. As can be seen from Figure 17B, there is a strong interaction between GST-16 E6 and His-E2, but no detectable binding of GST to His-E2. These

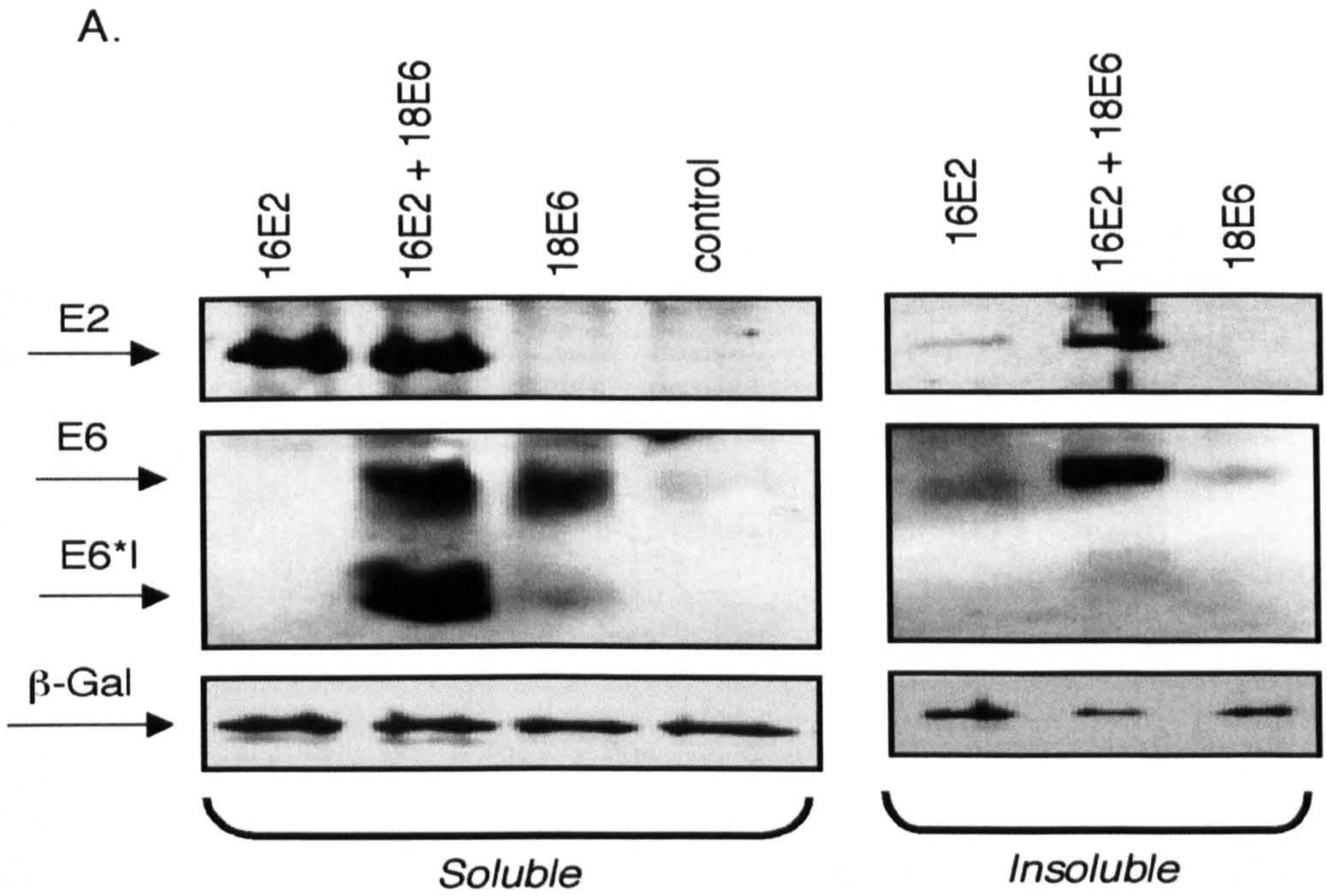


Figure 15. HPV E2 affects the ratio of E6/E6*I expression.
(A) U2OS cells were transfected with CMV-16 E2 and HA-tagged HPV-18 E6 either alone or in combination and after 24 hrs cells were harvested and analysed by western blot. The soluble and insoluble fraction of cells transfected as indicated were probed with anti-HA (12CA5, Roche) antibody to detect HA-18 E6/E6*I proteins and anti-E2 antiserum to detect E2. Anti-β-galactosidase (Promega) is used to check for equal transfection efficiency. (B) Comparison of HPV-18 E6/E6*I mRNA levels in U2OS cells transfected with plasmids expressing E6 alone or in combination with E2, analysed by RT-PCR amplification. The control for transfection efficiency was RT-PCR of GFP mRNA. The assay was repeated at least three times and equivalent results were obtained.

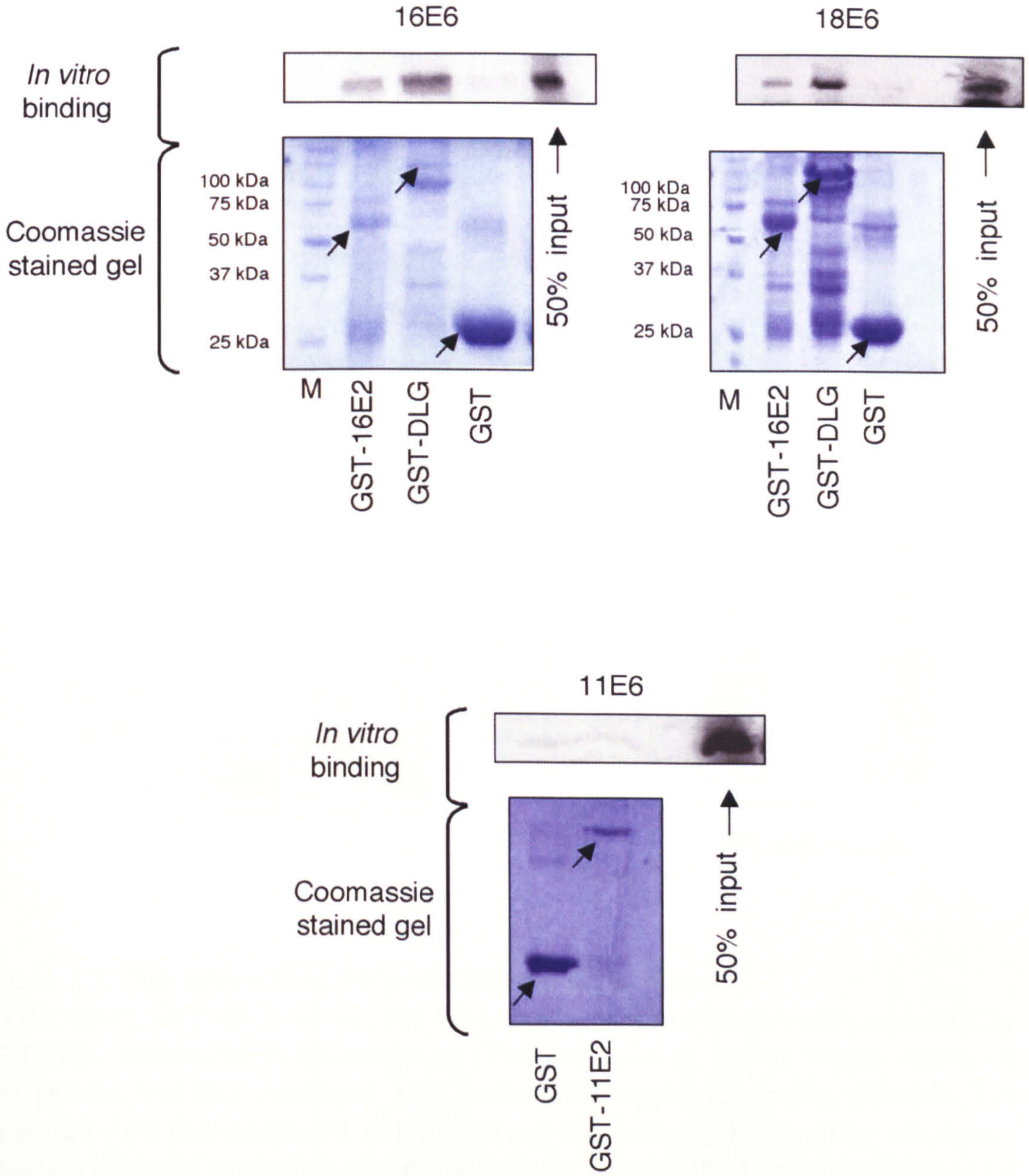


Figure 16. Sequences conserved between high-risk E6 proteins are important for binding to E2.

GST pulldown assays were performed with E6 proteins from both high-risk (16 E6 and 18 E6) and a low-risk HPV type (11 E6). GST-16 E2 protein was incubated with *in vitro* translated HPV-16 or 18 E6. The GST-Dlg and GST alone were included in the assay as positive and negative controls, respectively. For low-risk HPV, i*n vitro* translated HPV-11 E6 was incubated with GST-11 E2 or GST alone (negative control) and binding was assessed. The lower panels show the same gel rehydrated and stained with Coomassie Blue to control GST-fusion protein input. The Figure is the representative result of at least three different experiments.

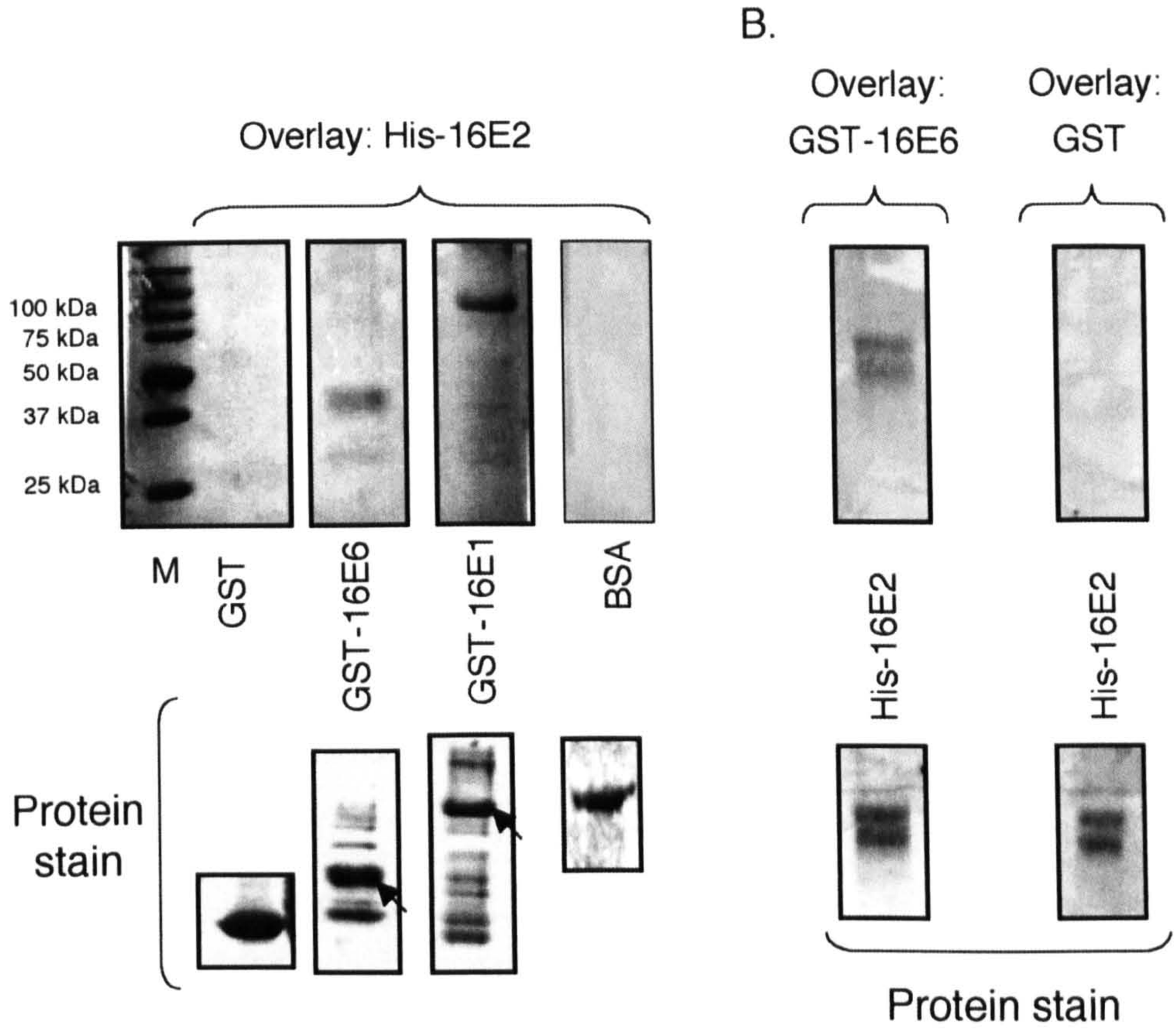


Figure 17. The interaction between E6 and E2 is direct.
(A) GST alone, GST-E6, GST-E1, and BSA (bovine serum albumin) were separated by SDS-PAGE, transferred to nitrocellulose (Ponceau stain of protein inputs shown on lower panels), and then incubated with purified His-tagged E2 protein (His-E2). The membranes were then incubated with mouse anti-His antibody followed by anti-mouse antibody conjugated to alkaline phosphatase (upper panels). (B) Purified His-tagged E2 protein was run on SDS-PAGE and transferred to nitrocellulose (Ponceau stain of protein inputs shown on lower panels) and then overlaid with either GST or GST-E6 fusion protein. After washing, the membrane was incubated with goat anti-GST antibody and then with anti-goat antibody conjugated to alkaline phosphatase (upper panel). Westerns were then developed using NBT/X-phosphatase solution (Boehringer Mannheim). The Figure is the representative result of at least three different experiments.

data demonstrate that the interaction between HPV-16 E6 and HPV-16 E2 is indeed direct.

Sequences within the carboxy terminus of E2 interact with E6

To determine which regions of E2 are required for binding to E6, a series of GST pulldown assays were performed by incubating the wild type GST-E6 protein with *in vitro* translated, radiolabelled full length E2, amino terminal E2 (amino acids 1-138) or carboxy terminal E2 (amino acids 237-365). The results obtained are shown in Figure 18A, where it is clear that E6 binds to sequences within the carboxy terminal half of E2. To further define the minimal region of E2 required for the interaction with E6, a series of E2 mutants progressively truncated from the carboxy terminus of the protein were produced, *in vitro* translated, and incubated with the GST-16 E6 resin. After extensive washing, the bound proteins were eluted and visualised by SDS-PAGE and autoradiography. A parallel binding assay was performed using GST-bound resin as a negative control. The results are shown in Figure 18B. As can be seen, high levels of binding to E6 are seen with the E2 fragments containing sequences from 202-347, 202-335 and 202-322. However, further truncations completely destroy the binding to E6. These results indicate that the region of E2 essential for binding to E6 lies within the sequence 306-322. A similar result was obtained using GST-18 E6 and the HPV-16 E2 mutants (Figure 18C), showing that the region of E2 essential for binding to both high-risk oncoproteins lies predominantly within the same sequence. As can be seen in Figure 18D, this region contains sequences necessary for E2 dimerization, but lies outside the core DNA binding domain of the protein (Hedge et al., 1992; Hegde and Androphy, 1998).

Residues lying between amino acids 28 and 31 are required for HPV-18 E6 binding to E2

In order to identify the region of E6 binding to E2 we made use of a previously characterized panel of HPV-18 E6 mutant proteins (Pim et al., 1994), which are shown schematically in Figure 19A. The various E6 mutants were translated *in vitro* and GST pulldown assays were

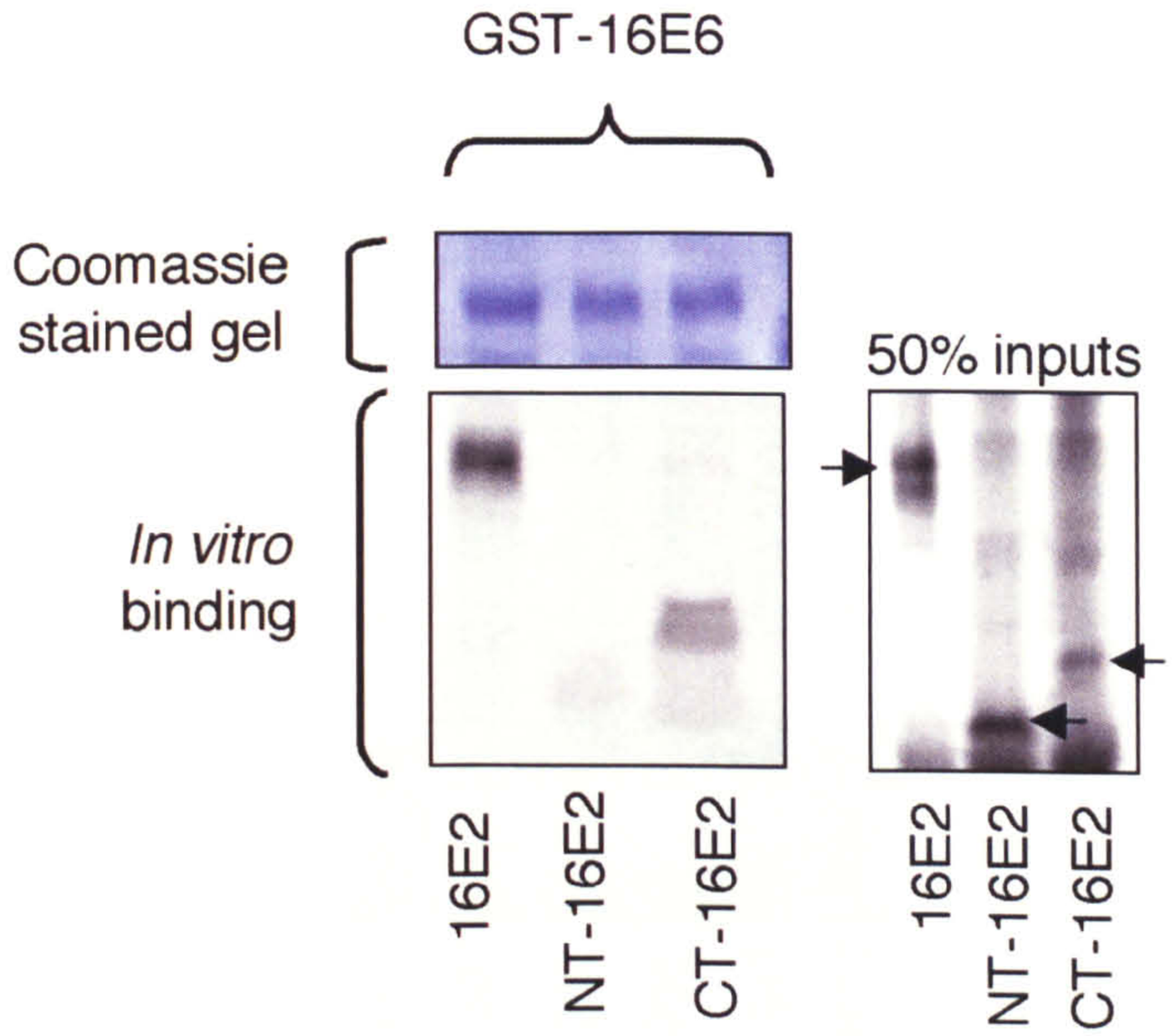
done using a GST-16 E2 fusion protein plus GST-16 E7 as a negative control. The results obtained are shown in Figure 19B. As can be seen, the three carboxy-terminal mutants of E6; ΔB (Δ94-98), ΔG (Δ126-130) and ΔH (Δ144-149) are all bound by E2 in a manner similar to wild type 18E6, as are the amino terminal mutants; ΔNT (Δ4-7) and ΔI (Δ36-39). In contrast, the amino terminal mutant, ΔM (Δ28-31), completely failed to bind to E2 in this assay. These results demonstrate that the region of E6 bound by E2 falls within amino acid residues 28-31.

Having identified the site of E2 interaction on E6, we then wanted to investigate whether the ability of E2 to alter E6's subcellular localisation is indeed due to its ability to bind to E6. Therefore, we repeated the immunofluorescence analysis with E6, E2 and the E6 ΔM mutant. U2OS cells were transfected as previously described, and the results obtained are shown in Figure 20A. As can be seen, the E6-ΔM mutant alone, like the wild type HPV-18 E6 (Figure 11A), shows a diffuse pattern of expression, with both nuclear and cytoplasmic staining. However, unlike wild type E6, the localisation of the E6-ΔM mutant does not change in the presence of E2 (Figure 20A). This result demonstrates that the interaction between E6 and E2 is necessary for the ability of E2 to relocalise or retain E6 in the nucleus.

The above results could also be explained, in part, by differences in the levels of expression of the wild type and ΔM mutant E6 proteins. In order to determine levels of expression of both E6 proteins, U2OS cells were transfected with plasmid constructs expressing wild type HA-tagged HPV-18 E6 or the mutant HA-tagged HPV-18 E6 ΔM along with an EGFP expressing construct used to check for equal transfection efficiency. Cells were harvested after 24 hrs and the level of each E6 protein was measured by western blot analysis using an anti-HA antibody. The results obtained are shown in Figure 20B and demonstrate similar levels of E6 ΔM expression to that of the wild type E6.

Since the region of E6 that is required for the interaction with E2 is also present within the spliced E6*I product, it was possible that E6*I might interact with E2. This seemed particularly relevant since we had also observed an increase in E6*I levels in the presence of E2 *in vivo* (Figure 15A). Therefore, to investigate this further, pulldown assays were performed with GST-

A.



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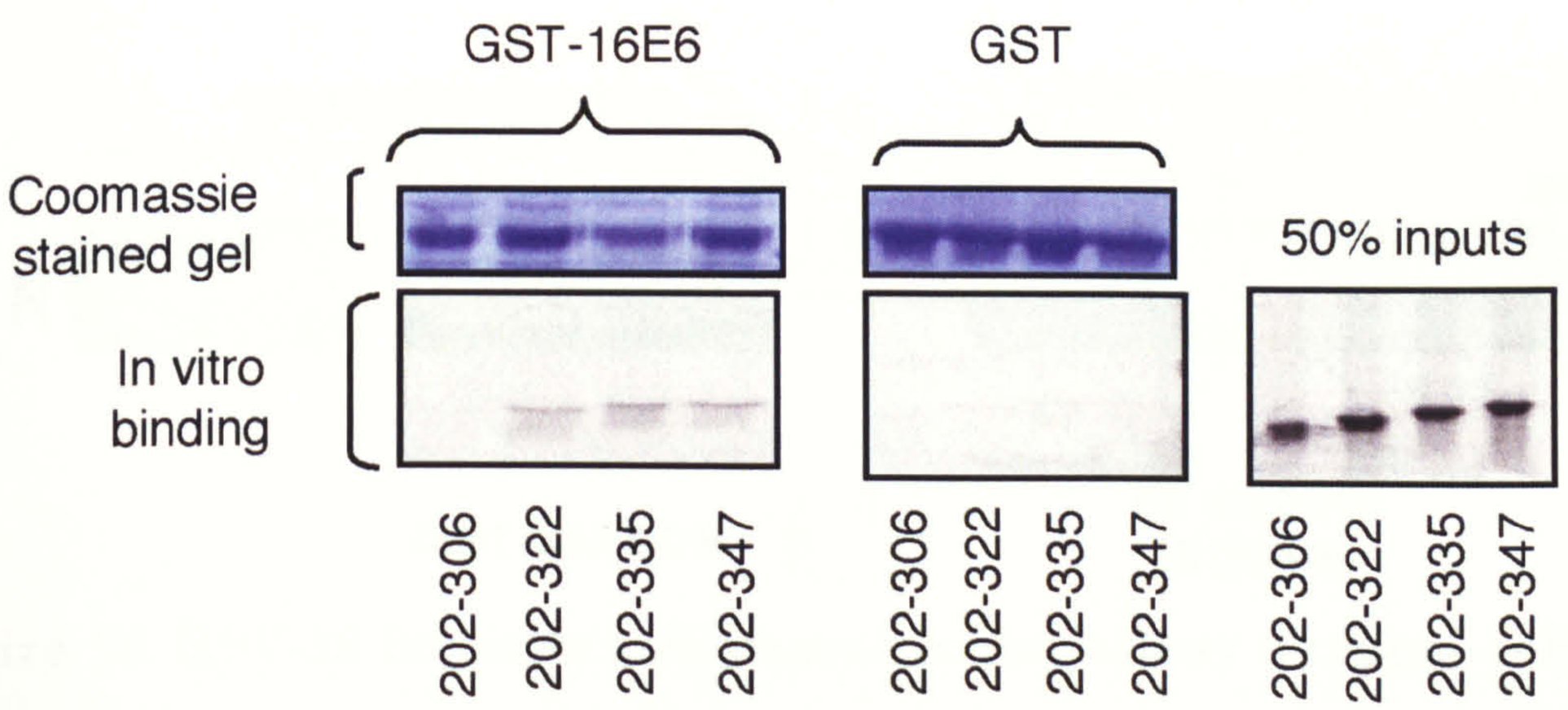
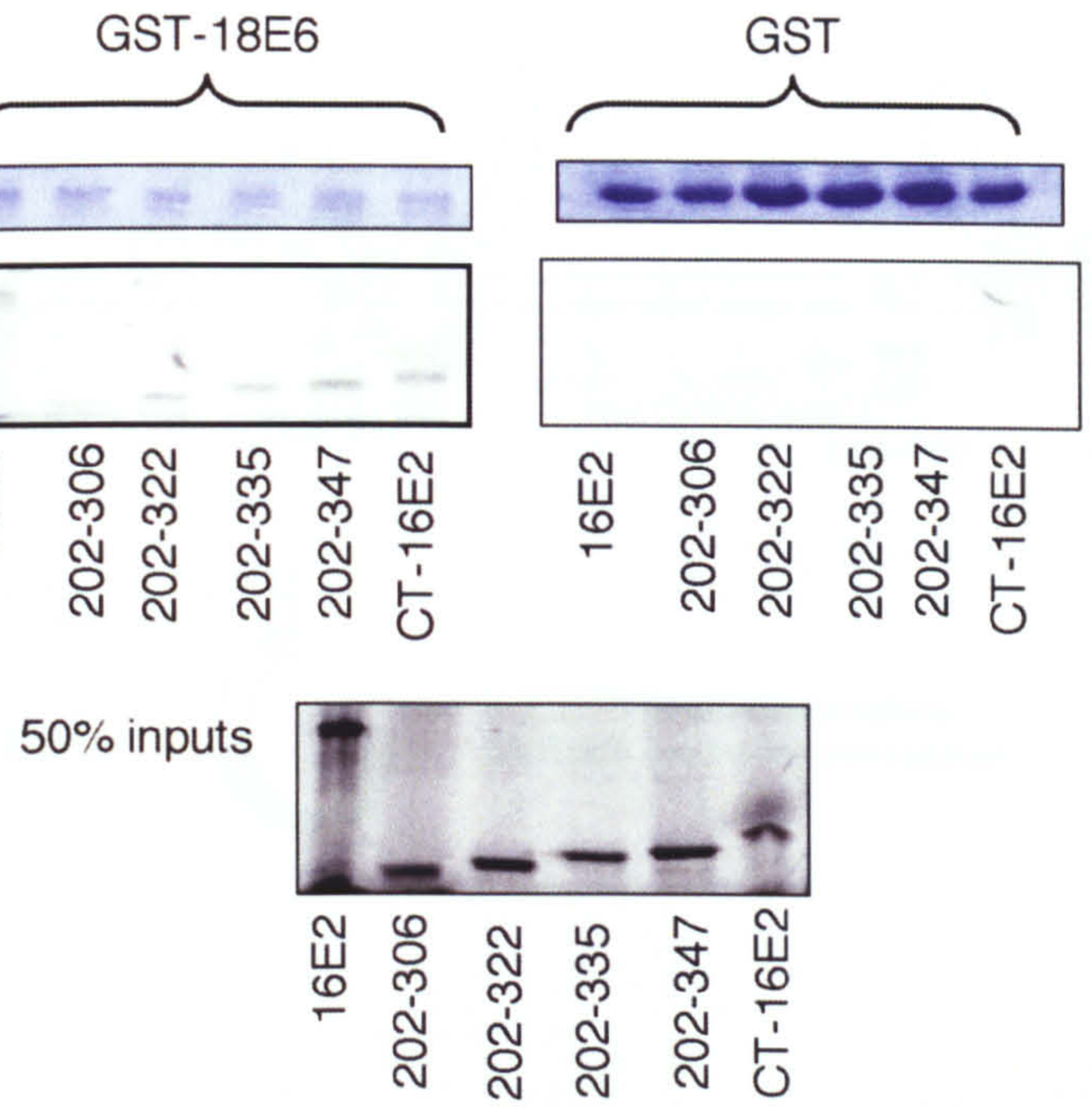


Figure 18. HPV-16 E6 binds to sequences in the carboxy terminus of HPV-16 E2.

(A) Purified GST-16 E6 was incubated with *in vitro* translated radiolabelled E2 proteins encoding either the full length E2 (16E2), amino terminal sequences from 1-138 (NT-16E2) or carboxy terminal sequences from 237-356 (CT-16E2). The bound proteins were then assessed by SDS-PAGE and autoradiography. Inputs for each E2 protein are shown in the right panel. The Coomassie stained gel shows the inputs of GST-16 E6 fusion proteins in top panel. (B) Identification of the minimal region of HPV-16 E2 required for binding to HPV-16 E6. A GST pull down assay was performed with GST-16 E6 fusion protein or GST alone using the *in vitro* translated E2 mutants as indicated. Bound E2 proteins were assessed by SDS-PAGE and autoradiography. Inputs (lower right panel) and Coomassie stained gels of GST-fusion proteins (top panels) are also shown.

C.



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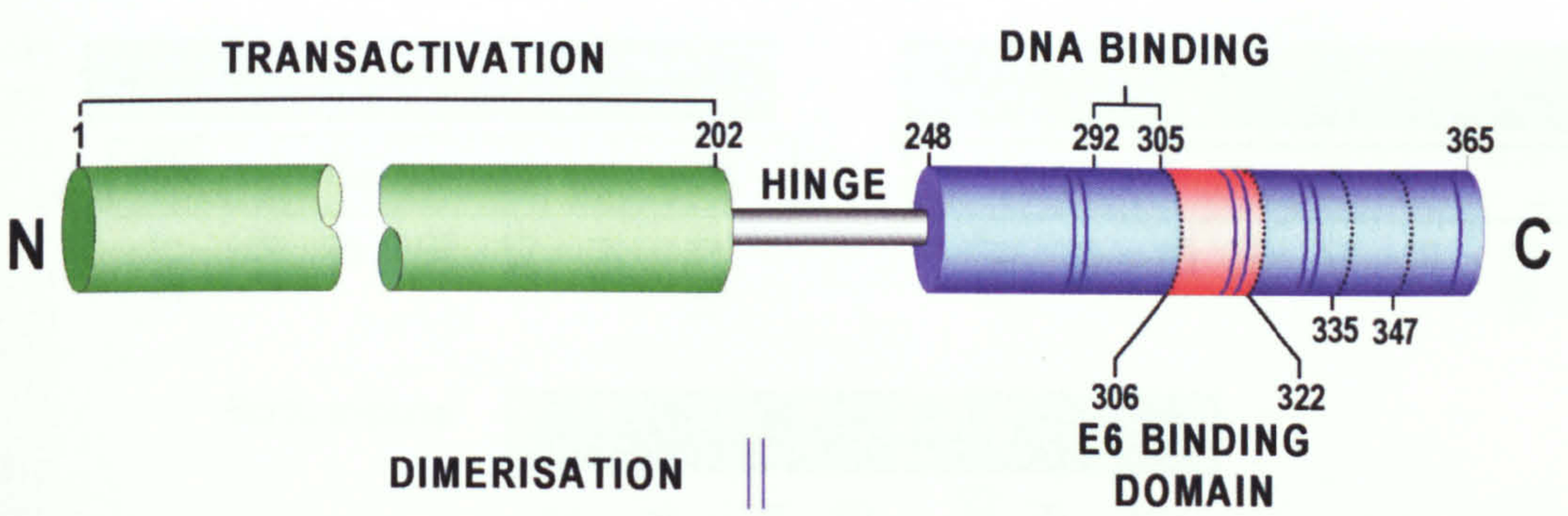
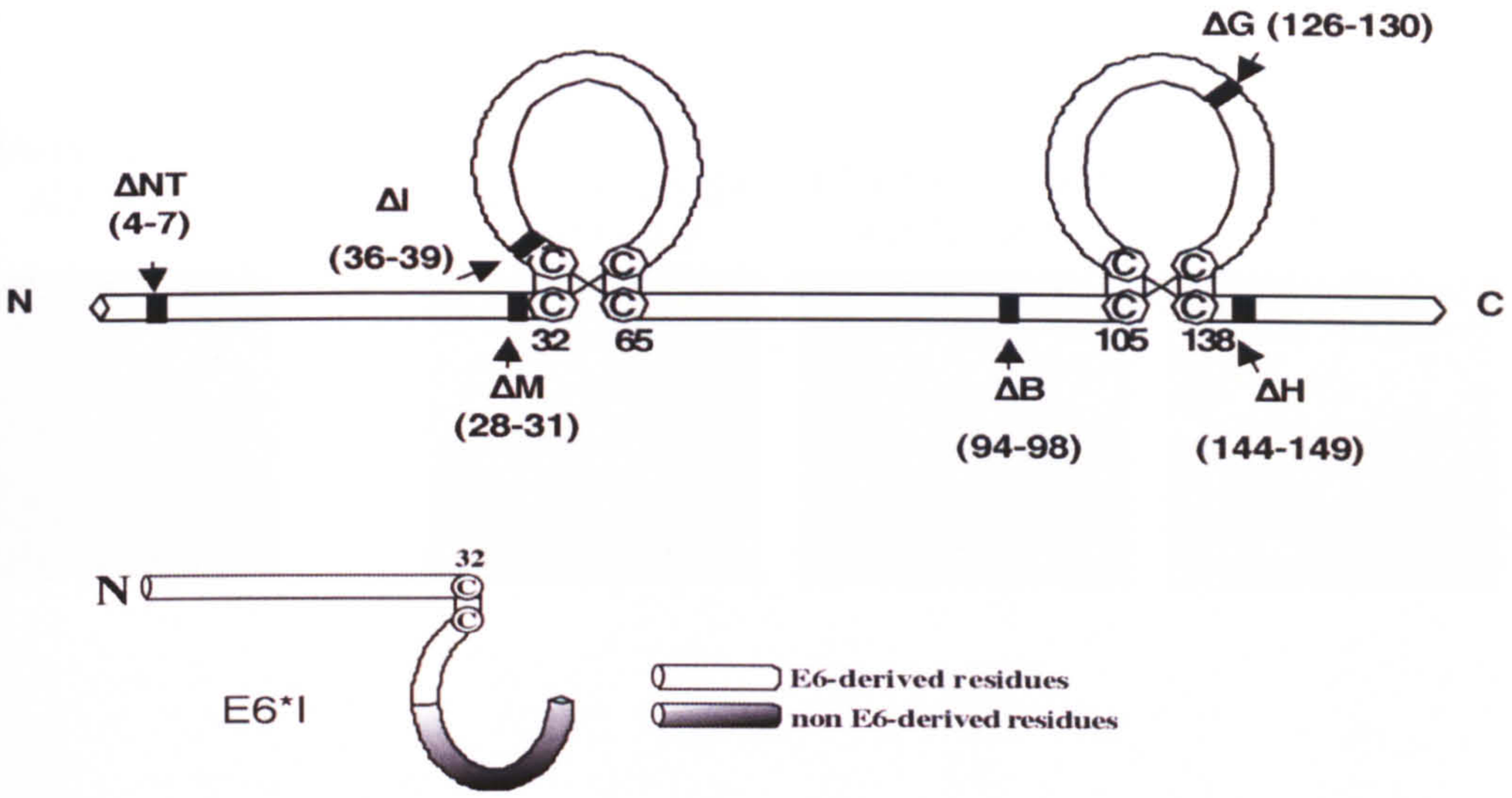


Figure 18. HPV-16 E6 binds to sequences in the carboxy terminus of HPV-16 E2.

(C) Identification of the minimal region of E2 required for binding to HPV-18 E6. A GST pulldown assay was performed with GST-18 E6 fusion protein or GST alone using the *in vitro* translated E2 mutants as indicated. Bound E2 proteins were assessed by SDS-PAGE and autoradiography. Inputs (lower panel) and Coomassie stained gels of GST-fusion proteins (top panels) are also shown. (D) Schematic diagram showing the location of the different E2 carboxy terminal truncated mutants used in the above mutational analysis and the domain identified as being responsible for the interaction with E6 (E6-binding domain). The domains important for known E2 functions are also indicated (the DNA binding domain is bracketed and those amino acid residues important for dimerisation are highlighted by double vertical lines) (Courtesy of D. Pim). All assays were repeated at least three times.

A.



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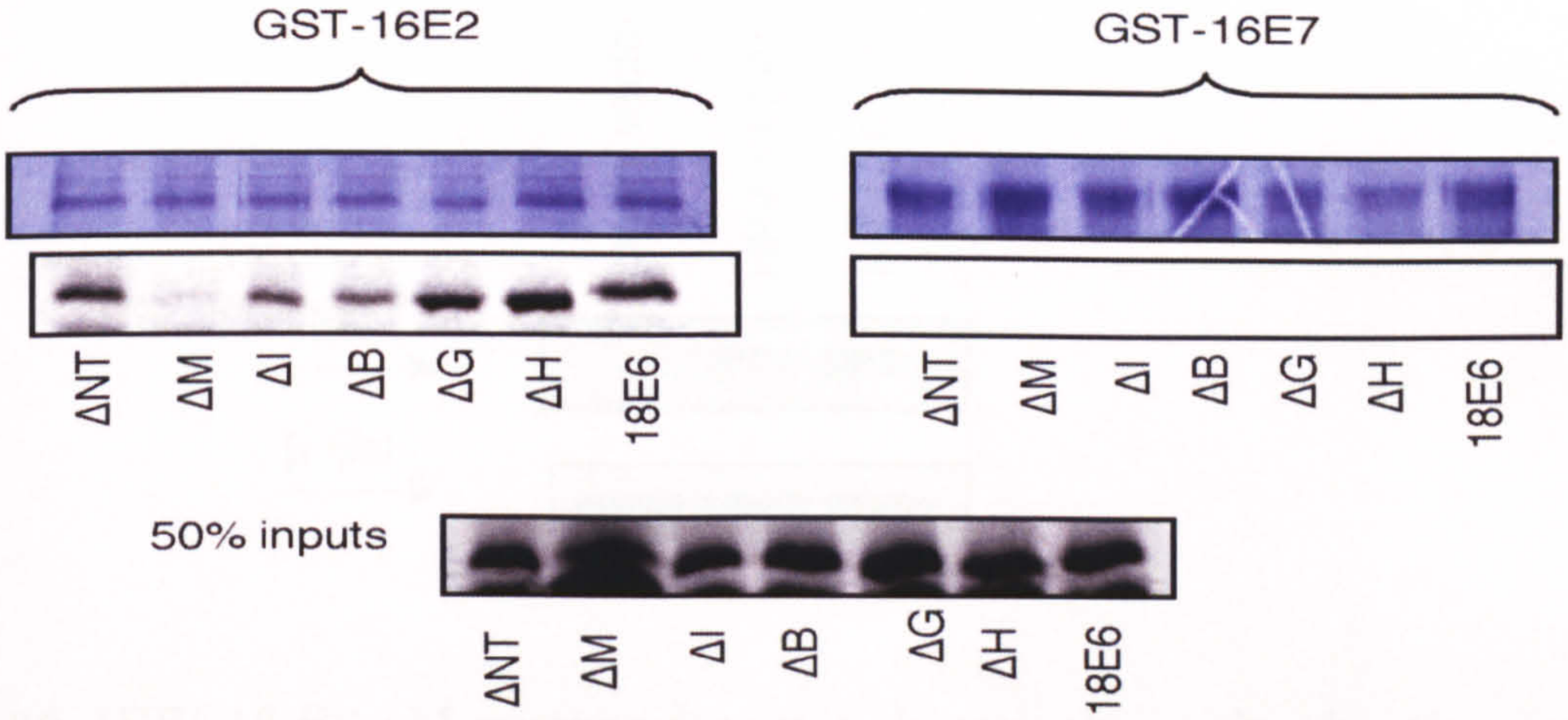
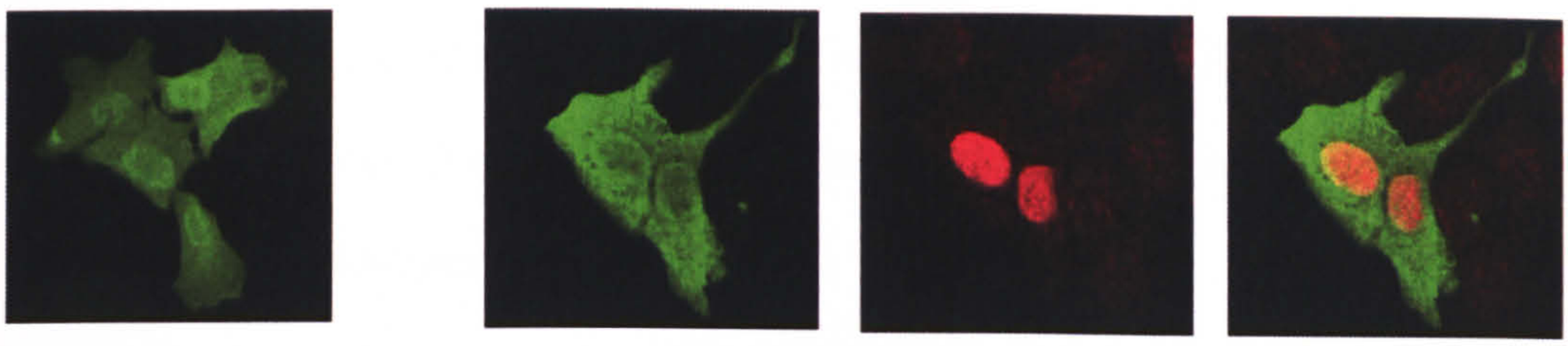


Figure 19. Identification of the E2 binding site on E6.
(A) Schematic representation of the HPV-18 E6 and E6*I proteins, together with the location of mutants used for the *in vitro* pulldown assays, ΔNT (Δ4-7), ΔM (Δ28-31), ΔI (Δ36-39), ΔB (Δ94-98), ΔG (Δ126-130), and ΔH (Δ144-149) (Courtesy of D. Pim). (B) *In vitro* translated wild type E6 and mutants were incubated with GST-16 E2 and with GST-16 E7 as a negative control. Bound E6 proteins were visualized using SDS-PAGE and autoradiography. After exposure the gels were rehydrated, then stained with Coomassie to show GST-fusion protein inputs (top panel). The inputs of each of the mutant E6 proteins is also shown (lower panel). The Figure shows the representative result obtained from at least three independent assays.

A.



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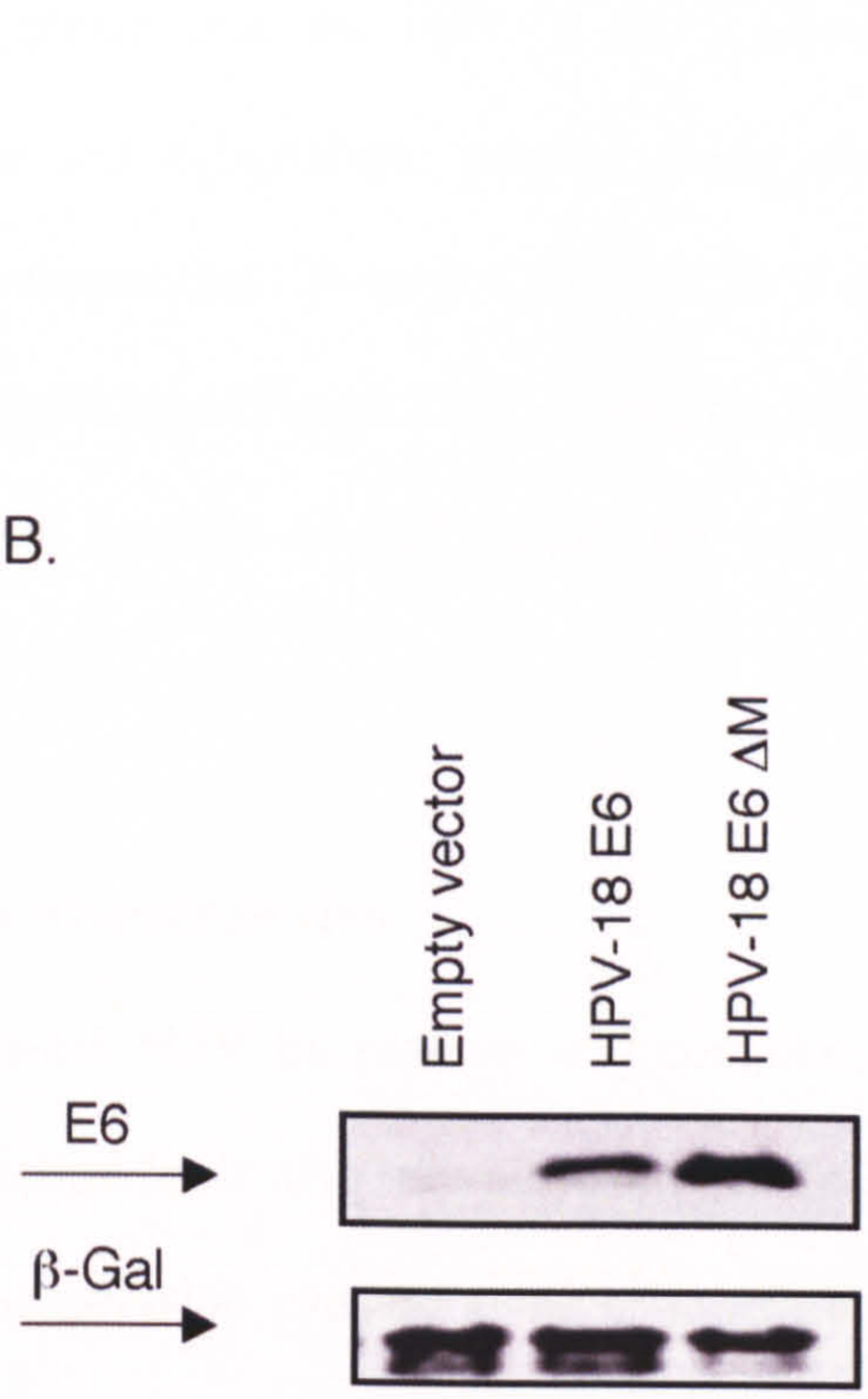


Figure 20. HPV-18 E6 ΔM mutant does not show nuclear relocalisation in the presence of E2.

(A) U2OS cells were transfected with a plasmid expressing the HA-tagged HPV-18 E6 ΔM mutant alone or cotransfected with a plasmid expressing HPV-16 E2, and the proteins were detected by double immunofluorescence, as indicated. The E6 ΔM mutant was detected with an anti-HA monoclonal antibody (Roche) and FITC-conjugated goat anti-mouse IgG; E2 was detected using anti-E2 antiserum, followed by rhodamine-conjugated goat anti-rabbit antibody. In each experiment a total of 30 fields were counted, and in this case out of 400 cells showing positive staining for both E2 and E6, none showed nuclear retention of E6. Similar results were obtained in each of three replicate experiments. (B) Comparison of wild type HA-18 E6 and mutant HA-18 E6 ΔM expression in transiently transfected U2OS cells. HA-E6 proteins were detected by anti-HA western blot. Probing of the western blot for β-galactosidase expression is shown in the lower panel and confirms equal levels of transfection efficiency.

18 E6*I (Pim et al., 1997), and *in vitro* translated E2 protein (Figure 21A). The ΔM deletion construct of E6*I (Pim & Banks, 1999) was also included in this assay. As expected, GST-18 E6*I efficiently bound *in vitro* translated E2 protein, whereas the GST-ΔM mutant of E6*I did not show any interaction, confirming the previous result (Figure 19B). Taken together, these studies demonstrate that both E6 and E6*I can interact with E2, and that this occurs through the same stretch of amino acids spanning residues 28-31.

Previous studies have shown that the HPV-18 E6*I protein gives a diffused pattern of expression with nuclear and cytoplasmic staining being visible (Guccione et al., 2004b). Surprisingly, when we coexpressed HA-tagged HPV-18 E6*I and E2 in U2OS cells, no change in E6*I localisation was observed (Figure 21B), raising the possibility that additional sequences in the carboxy terminal domain of full length E6 are necessary for the E2-mediated relocalisation.

HPV E2 and HPV E6 interact in vivo

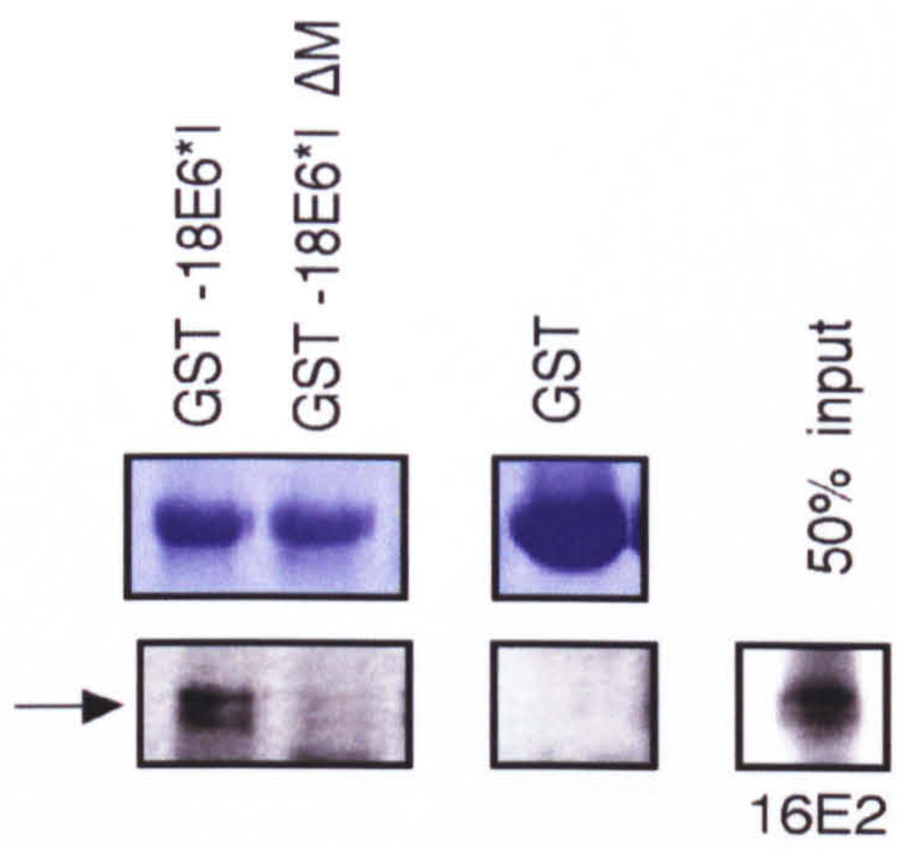
Having shown that high-risk HPV E6 proteins will complex with E2 *in vitro*, we wanted to know whether this interaction could also take place *in vivo*. To investigate this U2OS cells were transfected with the E2 expression plasmid alone or together with Flag-tagged E6 expression plasmids. After 24 hrs the proteins were extracted, immunoprecipitated with anti-Flag beads (Sigma) and the precipitated proteins were analysed by western blot with an anti-E2 polyclonal antibody (Massimi et al., 1999). As shown in Figure 22 the E6/E2 complexes was readily detected in cells cotransfected with Flag-E6 and E2 (Figure 22, lane 5) but not in cells transfected with E2 alone (Figure 22, lane 6), indicating that there is a specific *in vivo* interaction between the E6 and E2 proteins. Furthermore, in agreement with the results obtained from the *in vitro* binding assays, the ΔM-E6 mutant did not coimmunoprecipitate with E2, also showing that the region spanning residues 28-31 of E6 is necessary for the interaction with E2 *in vivo* (Figure 22, lane 4).

E2 inhibits E6-directed degradation of its PDZ-domain containing substrate proteins in vivo

Having demonstrated that E2 and E6 can interact *in vivo*, one consequence of which is relocalisation of the E6 protein, we were then interested in investigating whether this interaction could alter the efficiency by which E6 targets its substrate proteins for proteasome mediated degradation. To do this, we analysed three PDZ domain-containing substrates, MAGI-1, MAGI-2, and MAGI-3, together with the p53 tumour suppressor. For p53, Saos-2 cells were transfected with plasmids expressing p53, HPV-16 or HPV-18 E6 and HPV-16 E2. After 24 hrs cell extracts were made and p53 protein levels were ascertained by western blot analysis using a panel of anti-p53 monoclonal antibodies (Banks et al., 1986). The results obtained are shown in Figure 23A. As can be seen, the cotransfection of constructs expressing HPV-16 or HPV-18 E6 and p53 results in a significant decrease in the level of p53 protein. In addition, cotransfection of a construct expressing HPV-16 E2 together with the p53 and E6 had only a marginal affect on the levels of p53 degradation. Degradation assays performed *in vitro* also confirmed this data (Figure 23B).

We then investigated the effects of E2 upon E6-induced degradation of MAGI-1, MAGI-2 and MAGI-3. In this case, *in vivo* degradation assays were done in U2OS cells in the presence or absence of HPV-16 E2 expression plasmid. After 24 hrs cells were harvested and the levels of MAGI-1, MAGI-2 and MAGI-3 proteins were ascertained by western blot analysis. In agreement with previous observations, cotransfection of plasmids expressing HA-MAGI-1, HA-MAGI-2 or V5-MAGI-3 (Figure 24A) with plasmids expressing HPV-16 or HPV-18 E6 resulted in destabilization of the target protein (Figure 24A). Strikingly, however, in the presence of HPV-16 E2, the levels of MAGI-1, MAGI-2 and MAGI-3 were significantly stabilized (Figure 24A). Interestingly, E2 was unable to inhibit the E6-mediated degradation of MAGI-1 *in vitro* (Figure 24B), suggesting that the relocalisation of E6 by E2 may explain this observation. To further confirm this hypothesis we decided to use the E6 ΔM mutant, previously shown to be defective in binding E2, in these assays. To verify whether the E6 ΔM mutant

A.



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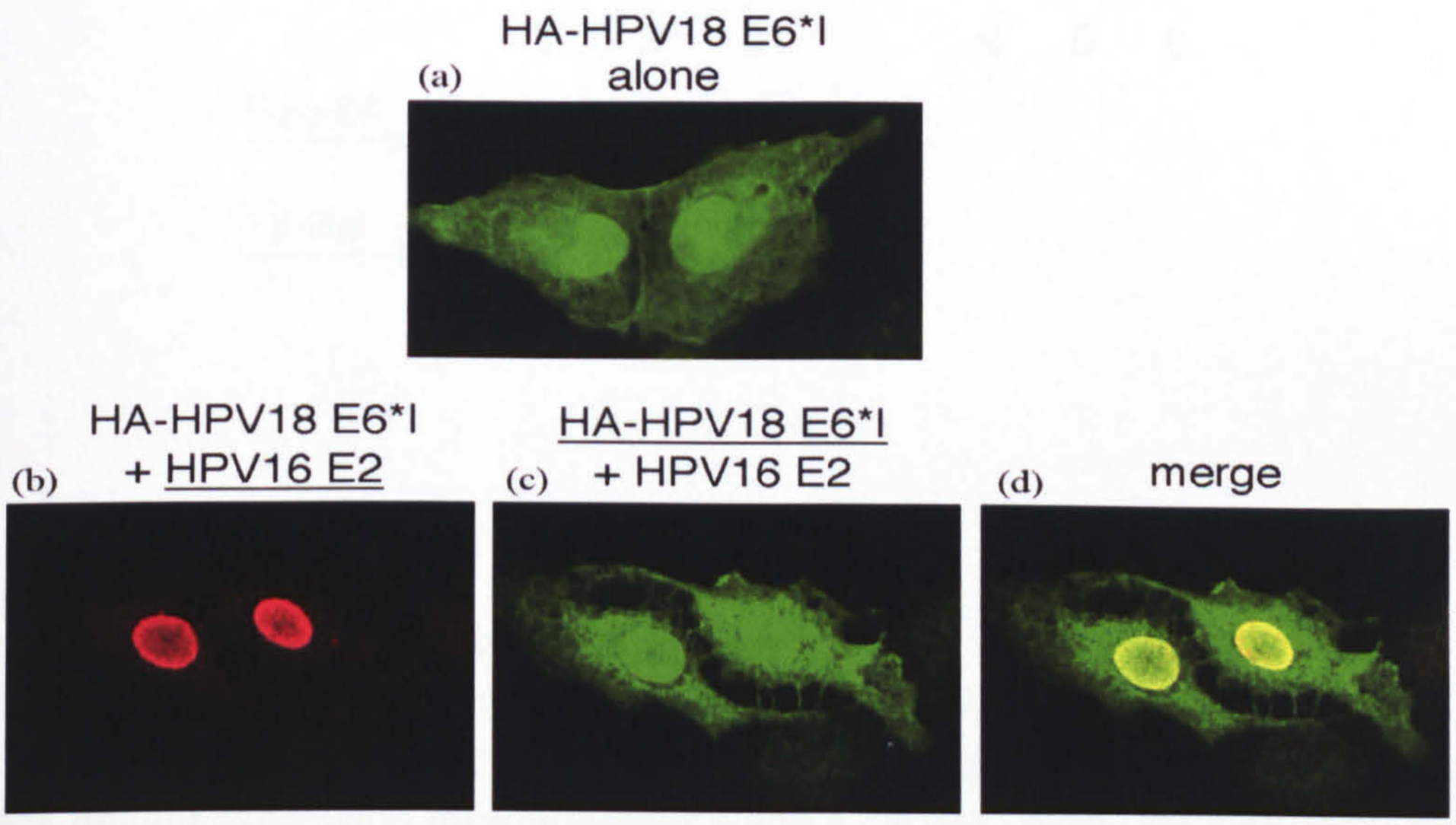


Figure 21. E2 interacts with the HPV-18 E6 spliced product E6*I.
(A) Radiolabelled *in vitro* translated E2 was incubated with GST-18 E6*I, GST-18 E6*I ΔM or with GST alone, as indicated. Bound E2 proteins were visualized using SDS-PAGE and autoradiography. After exposure gels were rehydrated, then stained with Coomassie to show GST-fusion protein inputs (top panel). The right-hand panel shows the input of E2. The assay was repeated at least three times and the figure shows a representative result. (B) Confocal analysis of the pattern of expression of HA-tagged HPV-18 E6*I and the HPV-16 E2 proteins. U2OS cells were transfected with a HPV-18 E6*I expression construct alone (a) or in combination with an HPV-16 E2 expression construct (b-d). Cells were stained with an anti-E2 polyclonal antibody and with an anti-HA mouse monoclonal antibody (12CA5, Roche). The stained proteins in double transfections are underlined. In each experiment a total of 10 fields were counted, and in this case out of 300 cells showing positive staining for both E2 and E6, none showed nuclear retention of E6. Similar results were obtained in each of three replicate experiments.

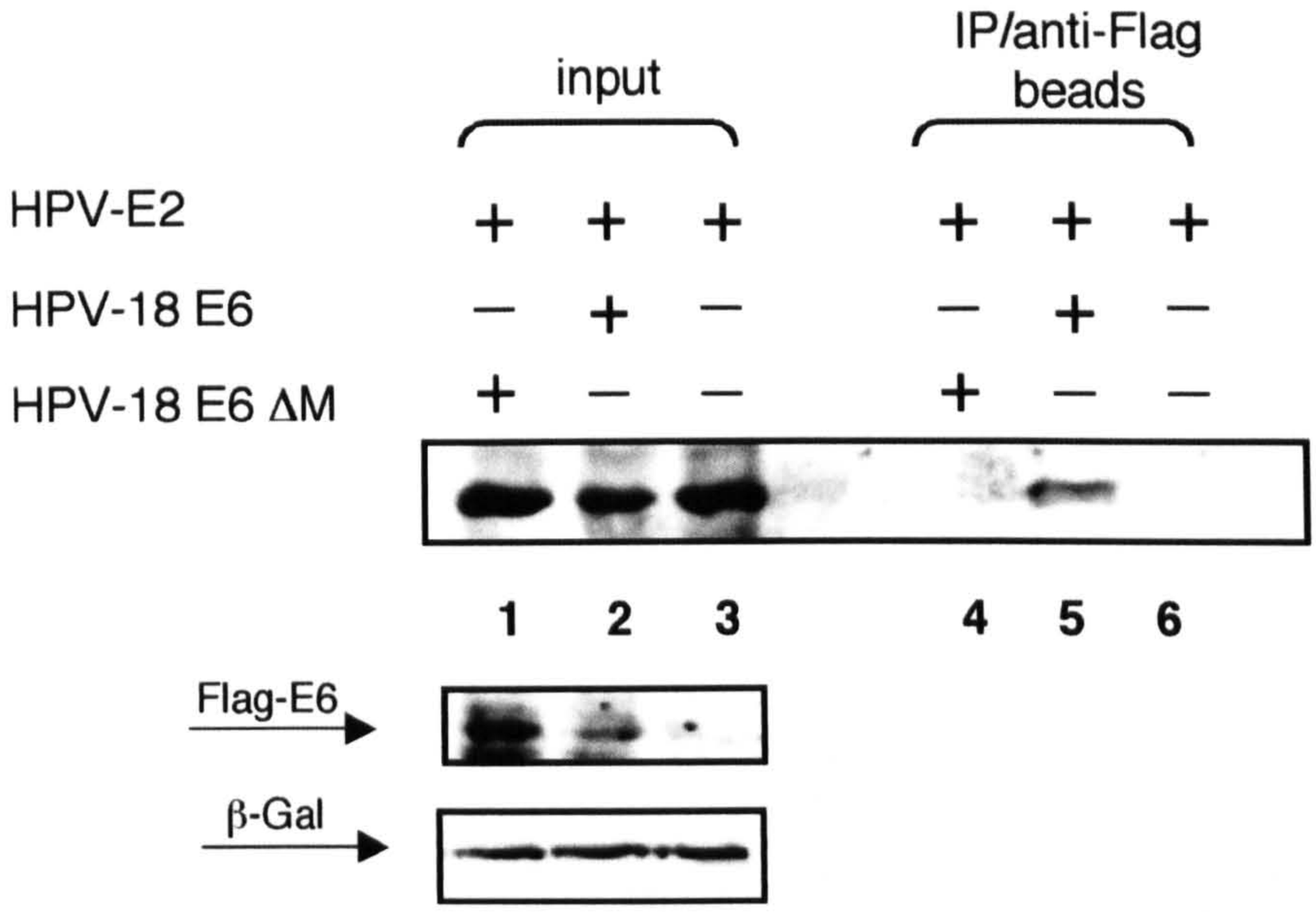
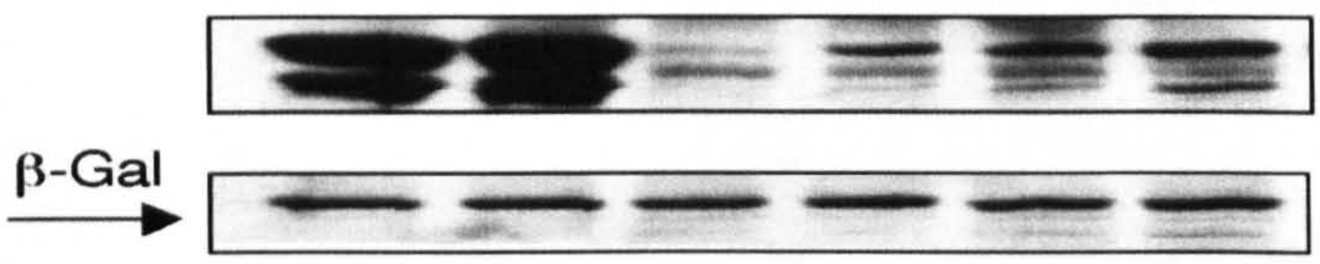


Figure 22. E2 interacts with E6 in vivo.
U2OS cells were transfected with either an E2 expression plasmid alone (lanes 3 and 6) or together with Flag-tagged wild type E6 expression plasmid (lanes 2 and 5) or Flag-E6 ΔM mutant expression plasmid (lanes 1 and 4). After 24 hrs, the cells were harvested and immunoprecipitated with the anti-Flag beads (M2, Sigma) (lanes 4-6). Coprecipitated E2 was detected by western blotting using an anti-E2 polyclonal antibody. Lanes 1-3 show 10% of the extract used for the immunoprecipitations. The bottom panel shows a western blot for β-galactosidase expression that was used as a control for transfection efficiency.

A.



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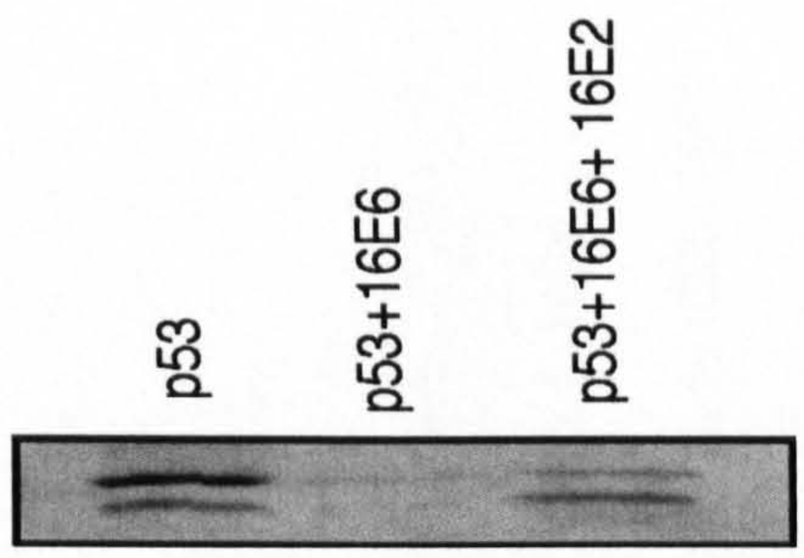


Figure 23. Effects of E2 upon E6-induced degradation of p53 in vivo.
(A) p53 null Saos-2 cells were cotransfected with p53 expression plasmid and HPV-16, -18 E6, and HPV-16 E2 expression plasmids, as indicated. After 24 hrs the cells were extracted and equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose. The p53 protein levels were ascertained by western blot analysis with a pool of anti-human specific p53 monoclonal antibodies (Banks et al., 1986). The lower panel shows the same blot reprobed for the cotransfected β-galactosidase (β-gal) to demonstrate equal levels of transfection efficiency (provided by L. Banks). (B) Effects of E2 upon E6-induced degradation of p53 *in vitro*. The *in vitro* translated HPV-16 E6 was pre-incubated with water primed lysate or HPV-16 E2 at 4°C for 30 min, prior to the addition of *in vitro* translated p53. The remaining target protein was detected by immunoprecipitation, followed by SDS-PAGE and autoradiography.

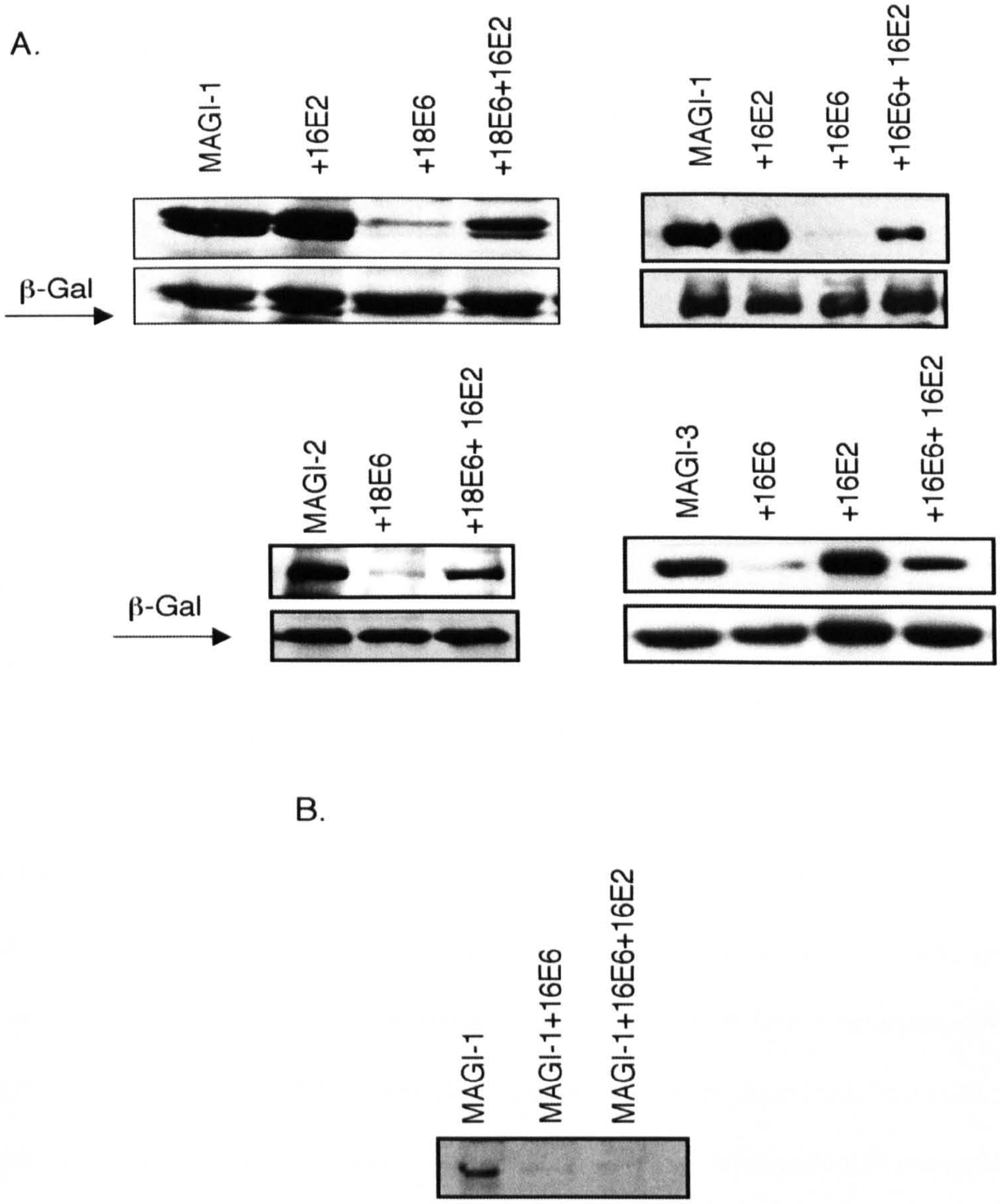


Figure 24. Effects of E2 upon E6-induced degradation of the MAGI proteins. (A) Extracts of U2OS cells transfected with MAGI-1, MAGI-2 or MAGI-3, with or without high-risk E6 and E2, as indicated, were analysed by western blot. Exogenous MAGI-1 and MAGI-2 proteins were detected using anti-HA antibody and MAGI-3 was detected using anti-V5 antibody. The lower panel in each case shows the same blot reprobed for the cotransfected β-galactosidase (β-gal) to demonstrate equal levels of transfection efficiency (part of the panel provided by L. Banks). (B) Effects of E2 upon E6-induced degradation of the MAGI-1 *in vitro*. The *in vitro* translated HPV-16 E6 was pre-incubated with water primed lysate or HPV-16 E2 at 4°C for 30 min, prior to the addition of *in vitro* translated MAGI-1. The remaining target protein was detected by immunoprecipitation, followed by SDS-PAGE and autoradiography. All assays were repeated at least three times and equivalent results were obtained.

retains its biological functions, we first performed an *in vivo* degradation assay with V5-tagged MAGI-3, together with wild type E6 or E6 ΔM mutant. After 24 hrs the cells were harvested and equalized samples of each extract were analysed for MAGI-3 protein levels by western blot with the anti-V5 antibody. The results obtained are shown in Figure 25A. As can be seen significant levels of MAGI-3 degradation were obtained with the ΔM mutant, thus demonstrating its functionality. This is consistent with a previous observation, which showed a substantial degradation of the Dlg protein induced by the E6 ΔM mutant (Pim et al., 2000). Having demonstrated that this mutant could direct the degradation of MAGI-3, we next investigated whether E2 could affect E6 ΔM activity and, as can be seen from Figure 25B, the ability of the mutant to degrade MAGI-3 is unaffected. This result further confirms that the effect of E2 upon E6's ability to degrade the MAGI proteins is mostly a consequence of its ability to bind E6.

Having found that E2 interferes strongly with the ability of E6 to degrade MAGI proteins, but only weakly with the degradation of p53, we wanted to determine whether this was simply due to differences in the efficiency with which E6 can degrade its different substrate proteins. To address this point, an *in vivo* degradation assay was performed, as described previously, with the difference being that U2OS cells were transiently transfected with plasmids expressing Flag-tagged p53 or HA-tagged MAGI-1, together with increasing concentrations of plasmids expressing HA-18 E6. After 24 hrs, the cells were harvested and the proteins extracted, and the amount of p53 or MAGI-1 was determined using an anti-Flag or an anti-HA antibody, respectively. The results are shown in Figure 26 and demonstrate that very low concentrations of HPV-18 E6 (1 μg) result in a significant decrease in the level of both p53 and MAGI-1, suggesting that E6 degrades p53 and MAGI proteins with similar efficiency. Hence the differences seen in Figures 23A and 24A are most likely due to the specific effects of E2 on E6 with respect to its ability to target the MAGI proteins and p53 for degradation.

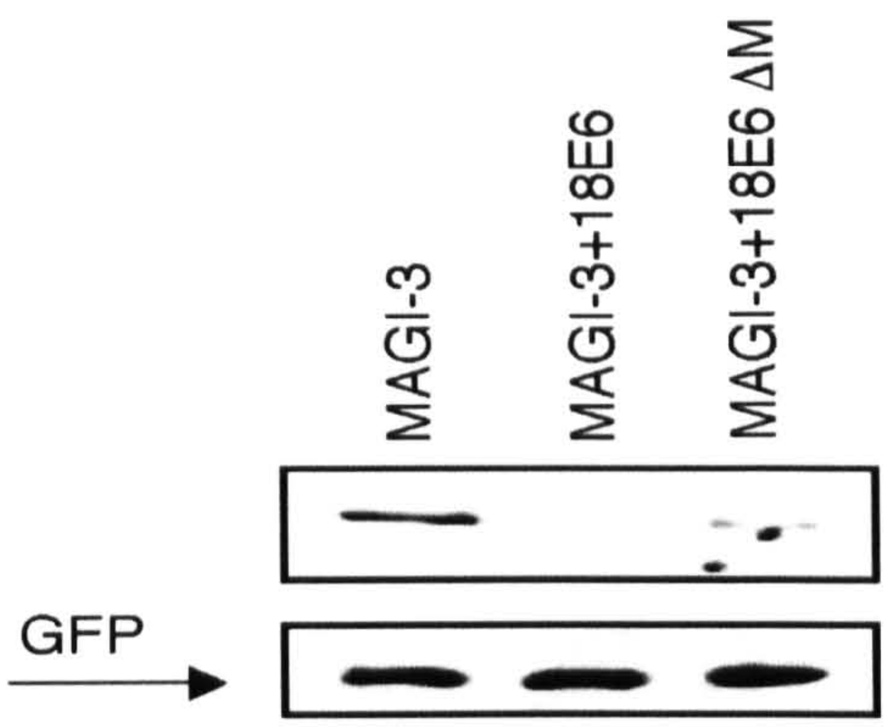
E6 stimulates E2-dependent transcription

Having shown that E2 can modulate E6's activities with respect to a number of its target proteins, we were next interested in investigating whether E6 could likewise modulate E2 function. The first activity we analysed was the ability of E2 to act as a transcriptional activator. Since previous studies have demonstrated an interaction between E6 and p300 (Patel et al., 1999) as well as between E2 and p300 (Lee et al., 2000a; Marcello et al., 2000), we used a mutant E6 protein (Δ123-127) which fails to bind p300, since use of a wild type E6 would render it unclear which effects were due to interaction with E2 and which due to interaction with p300. Furthermore, the assays were performed in Saos-2 cells to exclude any p53-related effects. The luciferase reporter plasmid (6xE2BS-Luc), containing 6 synthetic tandem repeats of the E2 DNA-binding site upstream of the luciferase gene, was cotransfected with the plasmids encoding E2 and the HPV-16 E6 Δ123-127 mutant into Saos-2 cells. After 24 hrs luciferase activity was measured and the results are shown in Figure 27A. As can be seen, E2 is a potent activator of transcription whilst E6 alone has no effect. However upon addition of E6 there is a modest stimulation of E2's transcriptional activity. To investigate whether this was due to increased DNA-binding activity of E2, a gel retardation assay was performed. As shown in Figure 27B, when labeled probe-containing an E2BS was incubated with E2 the E2-oligonucleotide complex migrated as a single discrete band. When *in vitro* translated E6 was added in the presence of E2, no change in DNA binding of E2 was observed (Figure 27B). The identity of these complexes was confirmed by a supershift analysis using anti-E2 antibodies. This result suggests that E6 does not stimulate E2-dependent transcription through an increased DNA binding activity of E2.

E6 inhibits E2-dependent DNA replication

Expression of HPV-16 E2 and E1 allows for efficient activation of replication from a plasmid containing the HPV-16 origin of replication (*ori*). To determine whether E6 can modulate this activity of E2, 293 cells were transfected with an HPV-16 *ori*-containing plasmid, together with

A.



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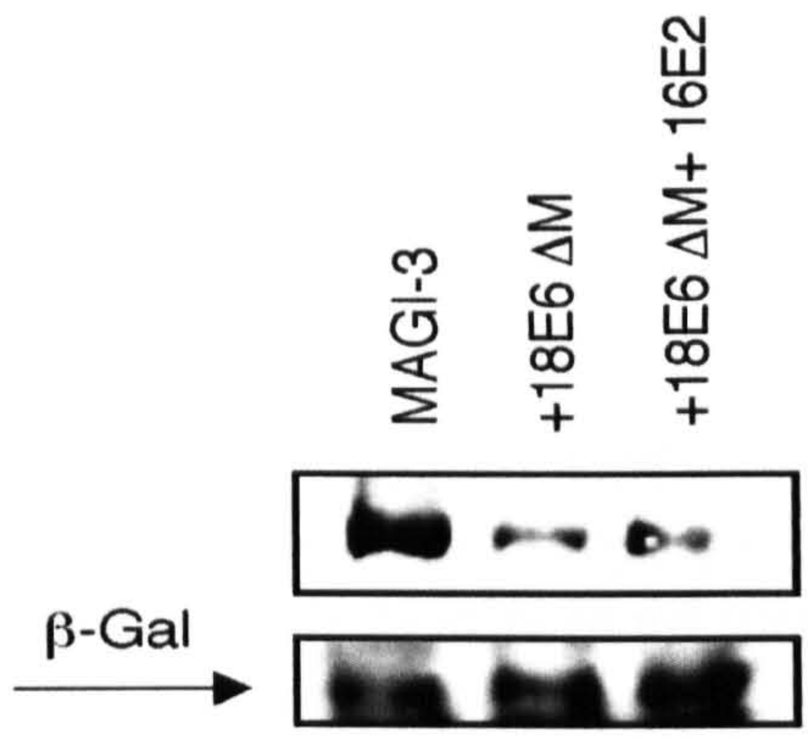
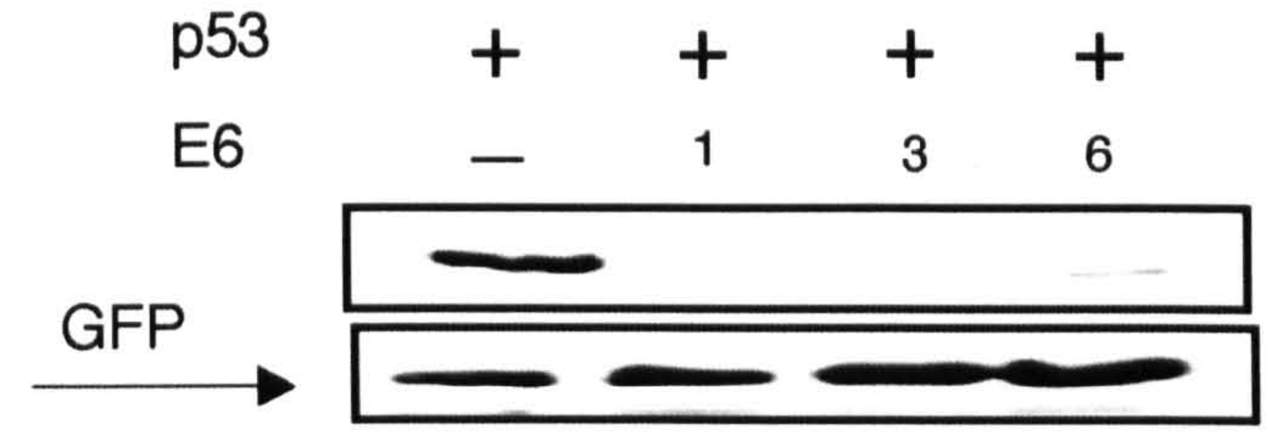


Figure 25. Inhibition of HPV-18 E6-induced degradation of MAGI-3 by E2 requires E6 binding activity.

(A) U2OS cells were transfected with 5 μg of the V5-MAGI-3 expression plasmid together with 5 μg of the wild type HA-18 E6 or mutant HA-18 E6 ΔM expression plasmids. After 24 hrs cells were harvested and residual MAGI-3 was detected by western blot analysis using an anti-V5 antibody. An EGFP expression plasmid was included as a control for transfection efficiency (lower panel). (B) U2OS cells were transfected with a V5-tagged MAGI-3 expression plasmid alone or with plasmids expressing the HA-tagged HPV-18 E6 ΔM mutant, or in combination with HA-18 E6 ΔM and HPV-16 E2, as indicated. After 24 hours, the proteins were extracted and the levels of MAGI-3 were determined by western blot analysis with an anti-V5 monoclonal antibody. β-Gal expression plasmid was used as an internal control to monitor the efficiency of transfection. The Figure shows the representative results of at least three different experiments.



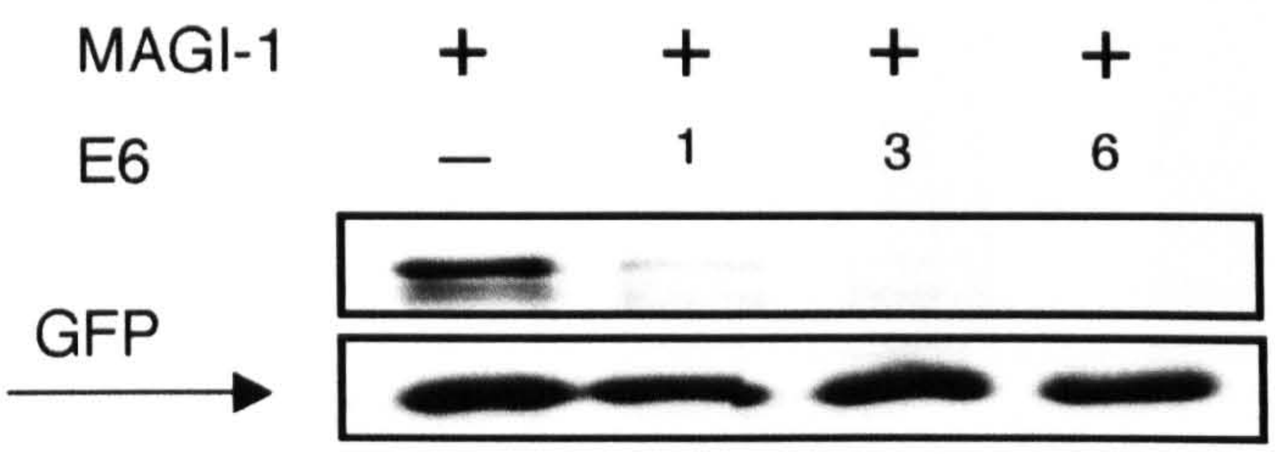


Figure 26. E6 degrades p53 and MAGI-1 with similar efficiency.
U2OS cells were cotransfected with Flag-tagged p53 expression plasmid (upper panel) or HA-tagged MAGI-1 expression plasmid (bottom panel) and the indicated amounts (μg) of HPV-18 E6 expression plasmid. After 24 hrs the cells were extracted and equal amounts of protein were separated by SDS-PAGE. The p53 and MAGI-1 protein levels were ascertained by western blot analysis with an anti-Flag antibody and an anti-HA antibody respectively. A GFP expression vector was used as a control to monitor the efficiency of transfection (lower panels). The Figure shows the representative results of at least three different experiments.

A.

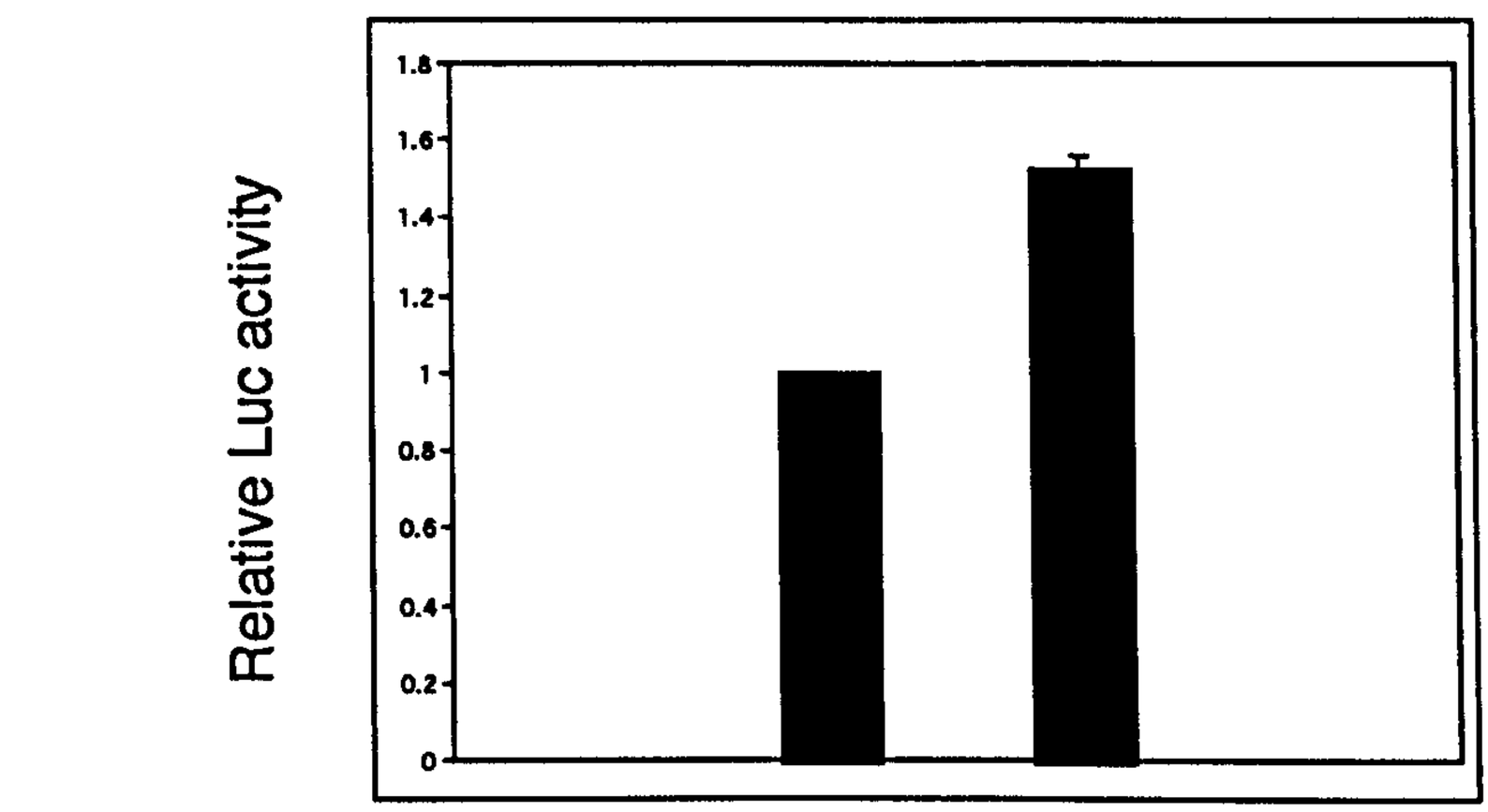


Figure 27. E6 modulates E2-dependent transcription.
(A) Saos-2 cells were cotransfected with a reporter construct (6xE2BS-Luc) containing six E2 binding sites upstream of the luciferase gene, an E2 expression vector and a vector encoding the HPV-16 E6 Δ123-127 mutant, as indicated. Cells were harvested after 24 hrs and the luciferase assay was performed. Representative results of three experiments are shown.

B.

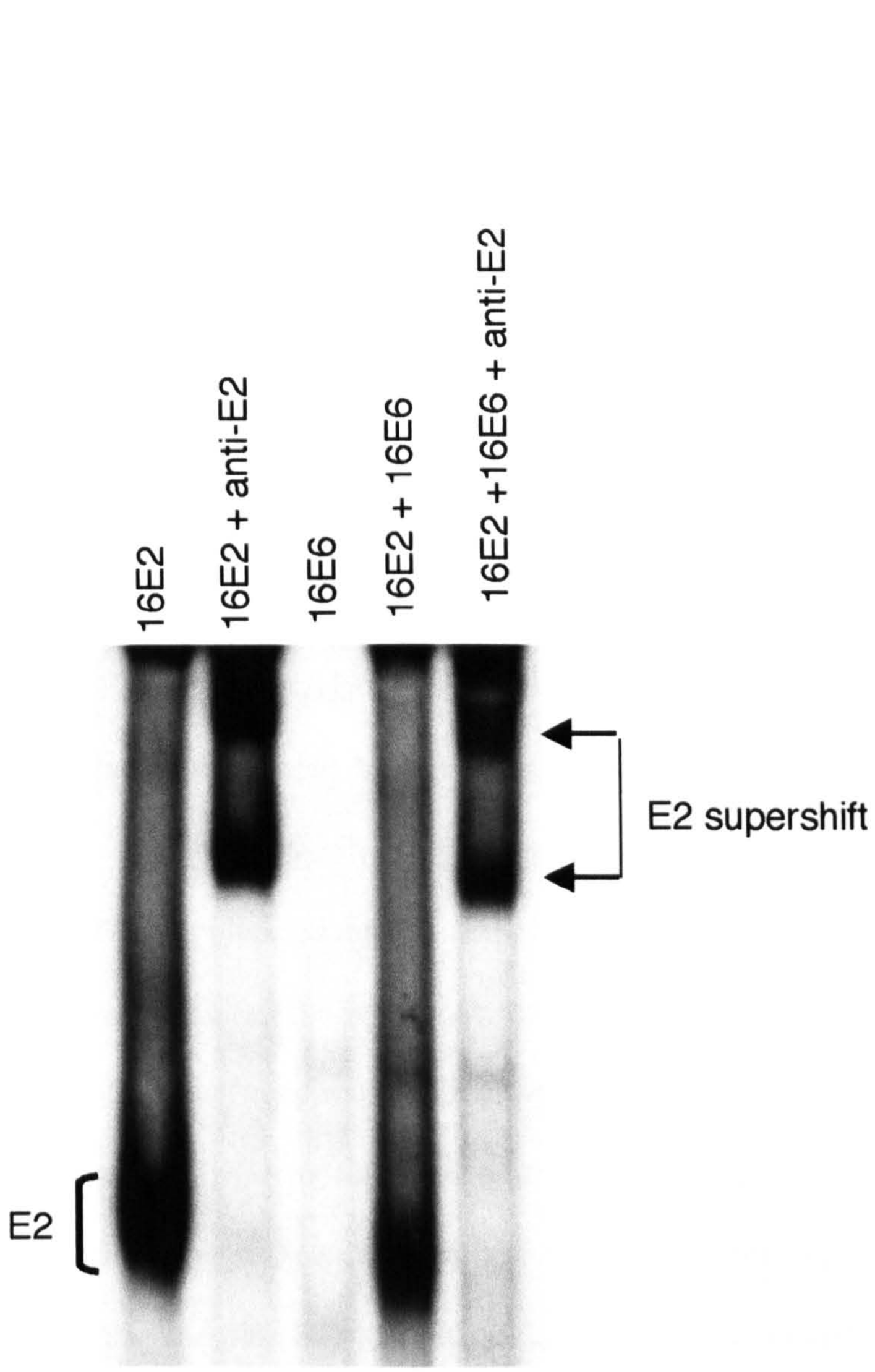


Figure 27. E6 modulates E2-dependent transcription.
(B) Gel retardation analysis of a labelled E2 recognition site oligonucleotide with *in vitro* translated E2 and E6 proteins. E2 and E6 were *in vitro* translated individually or together and incubated with a radiolabelled E2 recognition site oligonucleotide (E2BS). Complexes were separated on non-denaturing PAGE. Supershift can be seen with the proteins incubated with anti-E2 serum (Massimi et al., 1999), and retarded complexes corresponding to E2 are indicated. A representative result of three experiments is shown.

a plasmid expressing the HPV-16 E1 and E2 proteins, plus the wild type and selected mutant E6 proteins. The cells were harvested after 72 hrs and the amount of replicated plasmid DNA was assessed following *DpnI* digestion and southern blot analysis. The results obtained are shown in Figure 28. Cotransfection of the E1 and E2 expression plasmids with the HPV-16 *ori* plasmid resulted in robust replication (Figure 28A and B, lane 1). Coexpression of HPV-18 E6 strongly inhibited the HPV DNA replication in this system, and the same was true also for HPV-16 E6 (Figure 28A, lanes 2 and 3). The relationship between E6 interaction and E2 regulated DNA replication was characterized further by comparing wild type HPV-18 E6 function with the HPV-18 E6 ΔM mutant, defective in E2 binding. Since this mutant had no effect on the efficiency of HPV DNA replication in this assay (Figures 28A, lane 4 and 28B, lane 5), we conclude that binding to E2 is absolutely required for the ability of E6 to inhibit viral DNA replication. In order to further confirm the inhibitory effect of the E6 proteins on E2-dependent DNA replication, the assay was repeated using other E6 expression plasmids. The results are shown in Figure 28B. As can be seen, both the non-splicing mutant, expressing only full-length HPV-18 E6 (E6 NS), and the HPV-16 E6 Δ123-7 mutant, impaired in binding to p300, efficiently inhibited viral DNA replication (Figure 28B, lanes 3 and 6, respectively). Interestingly, the HPV-18 E6*I protein had no effect on viral DNA replication, even through it still retains the ability to bind E2 (Figure 28B, lane 4). Taken together these studies demonstrate that E6 is a potent inhibitor of E2-stimulated viral DNA replication, and that this most likely occurs through a direct interaction between E2 and E6.

E2 association with mitotic chromosomes is not perturbed by E6

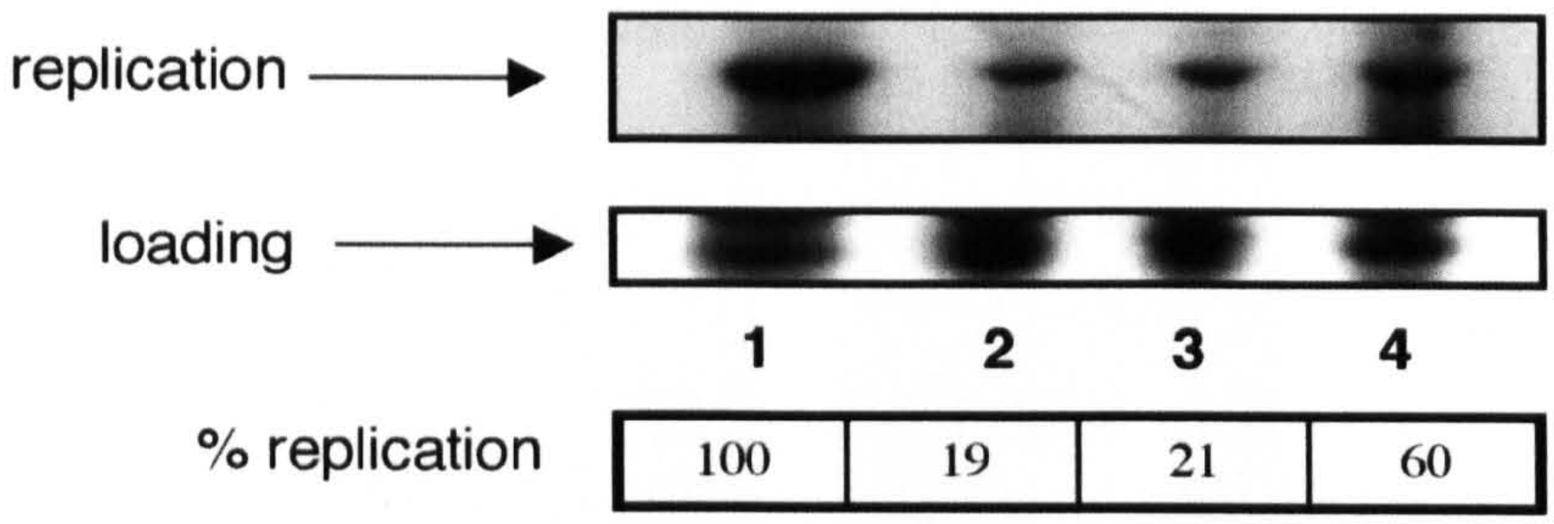
Several groups have shown that E2 facilitates viral genome segregation by interacting simultaneously with condensed mitotic chromatin and viral genomes (Skiadopoulos & McBride, 1998; Lehman & Botchan, 1998; Ilves et al., 1999; Bastien & McBride, 2000; Voitenleitner & Botchan, 2002; You et al., 2004). From these studies, the simple hypothesis arose whereby a cellular mitotic chromosomal protein binds to the activation domain of E2 and the plasmid DNA

hitchhikes onto chromosomes via binding to the DNA-binding domain of the viral E2 protein. Recently, a bromodomain protein Brd4 was identified as a major component of the E2 chromosomal attachment complex, thus confirming the hypothesis (You et al., 2004), To further address the potential biological significance of the E2/E6 interaction, we wanted to investigate whether this interaction would affect E2 mitotic chromosome association. Since all the studies described to date were done using BPV-1 E2, we first needed to assess whether HPV-16 E2 could also associate with mitotic chromosomes. To do this, U2OS cells were transfected with HPV-16 E2 and after 48 hrs cells were stained using an anti-E2 rabbit polyclonal antibody. To identify mitotic cells, the cellular DNA was labeled with Hoechst. The results are shown in Figure 29, and demonstrate that HPV-16 E2 does indeed associate with mitotic chromosomes, suggesting the existence of a common mechanism used by different papillomaviruses to segregate the viral genomes. However, it has been previously shown that BPV-E2 is associated with mitotic chromosomes at every stage of mitosis (Bastien & McBride, 2000). In contrast, we observed HPV-16 E2 associated with mitotic chromosomes only during cytokinesis, which begins in anaphase (Figure 29, panels c-d) and continues through telophase (Figure 29, panels e-g) until the formation of the midbody that is formed between the two daughter cells (Figure 29, panel h). As can clearly be seen from Figure 29, panels a-b, E2 is excluded from the mitotic chromosomes in metaphase. Taken together, these data suggest that different papillomaviruses may use E2 proteins to segregate viral genomes between the daughter cells, but that the precise mechanisms involved may differ.

Having shown that HPV-16 E2 can associate with mitotic chromosomes, we were then interested in determining whether the E6 protein had any influence upon this. U2OS cells were transfected with HA-tagged HPV-18 E6 and CMV-E2 expression plasmids and their respective patterns of localisation were determined by immunofluorescence, using an anti-HA or anti-E2 polyclonal antibody. As shown in Figure 30B, both E2 and E6 are excluded from mitotic chromosomes in metaphase (Figure 30B, panels a-c). However, in telophase the E2 is observed as a random speckled pattern associated with the chromosomes which is indistinguishable from

A.

HPV-16 URR	+	+	+	+
pCGE1BΔE5	+	+	+	+
HPV-16E6	—	+	—	—
HPV-18E6	—	—	+	—
HPV-18E6 ΔM	—	—	—	+



B.

HPV-16 URR	+	+	+	+	+	+
pCGE1BΔE5	+	+	+	+	+	+
HPV-18E6	—	+	—	—	—	—
HPV-18E6 NS	—	—	+	—	—	—
HPV-18E6*I	—	—	—	+	—	—
HPV-18E6 ΔM	—	—	—	—	+	—
HPV-16E6 Δ123-7	—	—	—	—	—	+

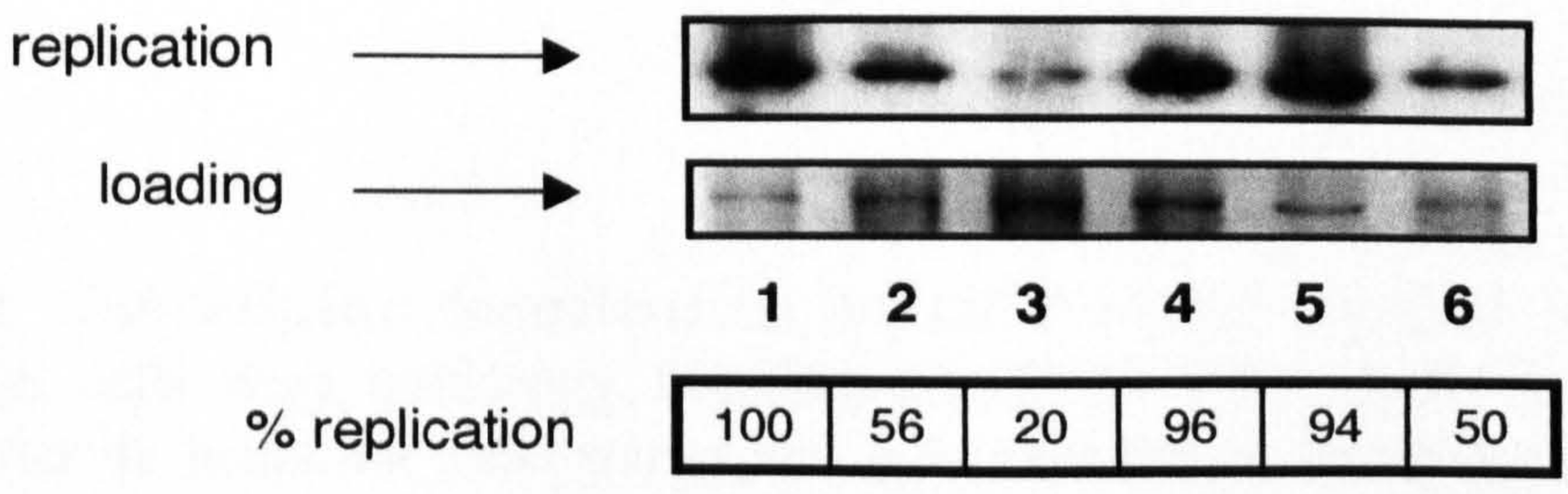


Figure 28. ***E6 inhibits E2-dependent viral DNA replication.***
293 cells were transfected with an HPV-16 *ori*-containing plasmid (HPV-16 URR) together with a plasmid expressing HPV-16 E1 and HPV-16 E2 (pCGE1BΔE5), as well as the indicated E6 expression plasmids. Low-molecular-DNA was digested with *DpnI*, and replicated DNA was detected by southern blot hybridisation using a high specific activity probe (upper panels in A and B). An Ethidium Bromide-stained agarose gel before blotting on to nylon membrane is also shown as a loading control (panel B, lower panel). The numbers below each lane show the percentage of viral DNA replication, with E1 and E2 plus the *ori* plasmid being normalised to 100%.

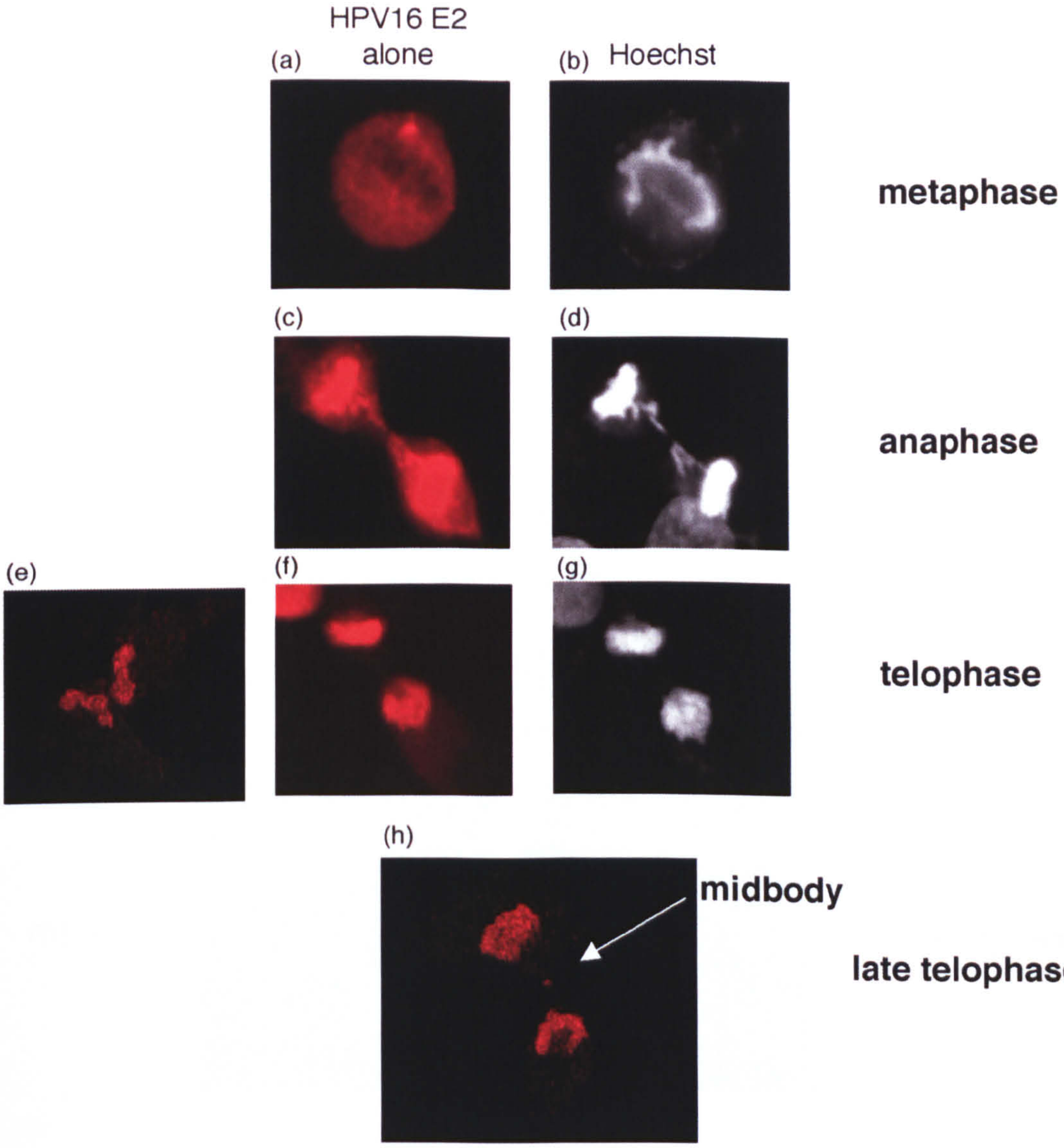


Figure 29. ***Subcellular localisation of HPV-16 E2 during the cell cycle.*** U2OS cells were transiently transfected with the HPV-16 E2 expression construct. After 48 hours, the cells were fixed and stained for HPV-16 E2 (primary: rabbit polyclonal anti-E2; secondary: rhodamine-red anti-rabbit, Molecular probes). Representative cells from different stages of mitosis (metaphase, anaphase, telophase) are depicted. The panels a, c, e, f and h show E2 (red) staining, and the panels b, d and g show Hoechst-stained DNA images. The E2 association with mitotic chromosomes in anaphase and telophase was observed in 100% mitotic cells expressing E2 (n=200), analysed in five different experiments.

A.

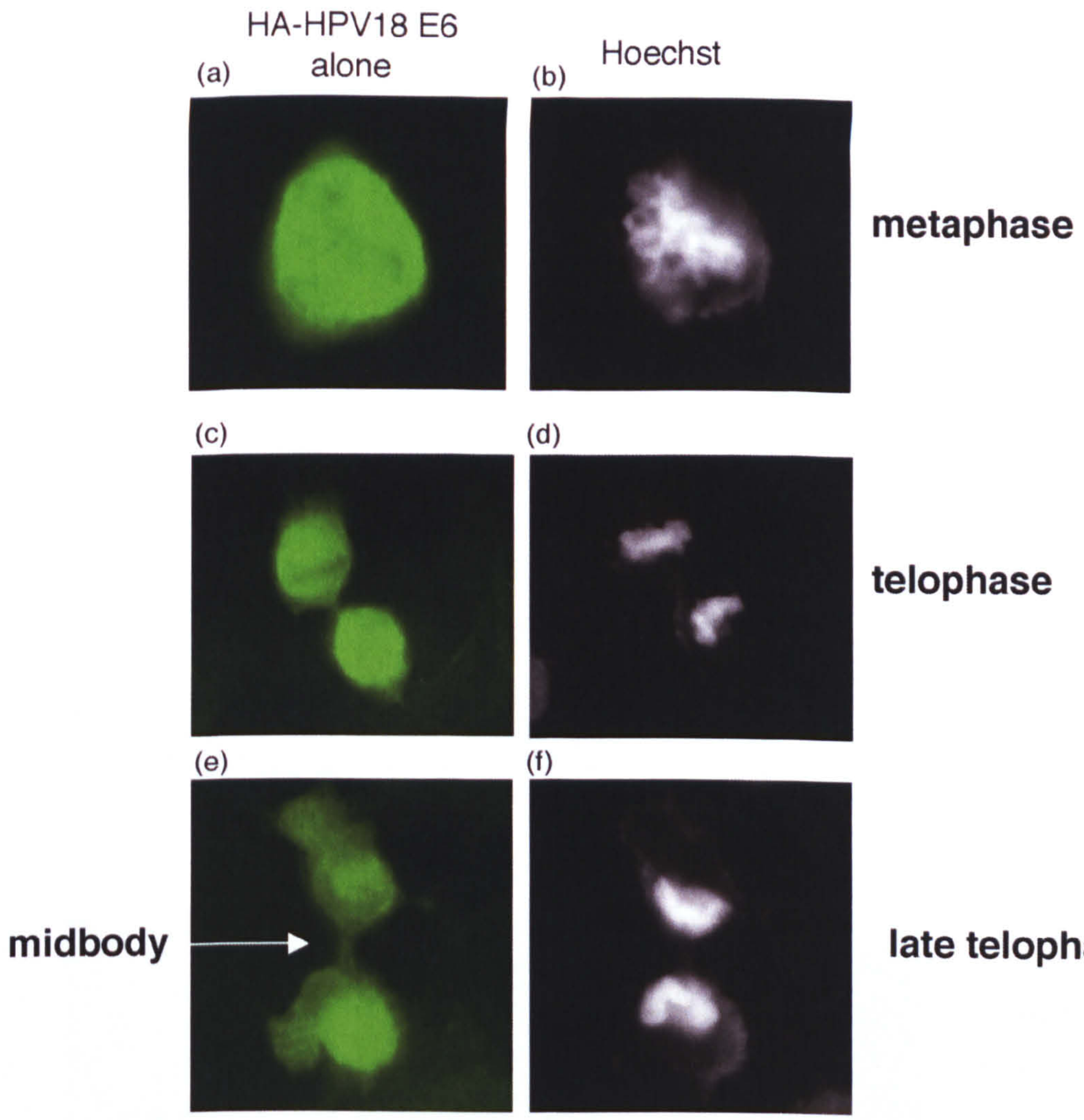


Figure 30. HPV-16 E6 does not associate with mitotic chromosomes in transiently transfected U2OS cells.

(A) Cells transfected with the HA-18 E6 expression construct were fixed and stained for HA-18 E6 (panels a, c and e) using an anti HA mouse monoclonal antibody (green, 12CA5, Roche). Different stages of mitosis were determined using Hoechst staining (panels b, d and f). No association of E6 with mitotic chromosomes was observed in any of the positive staining cells analysed (n=200).

B.

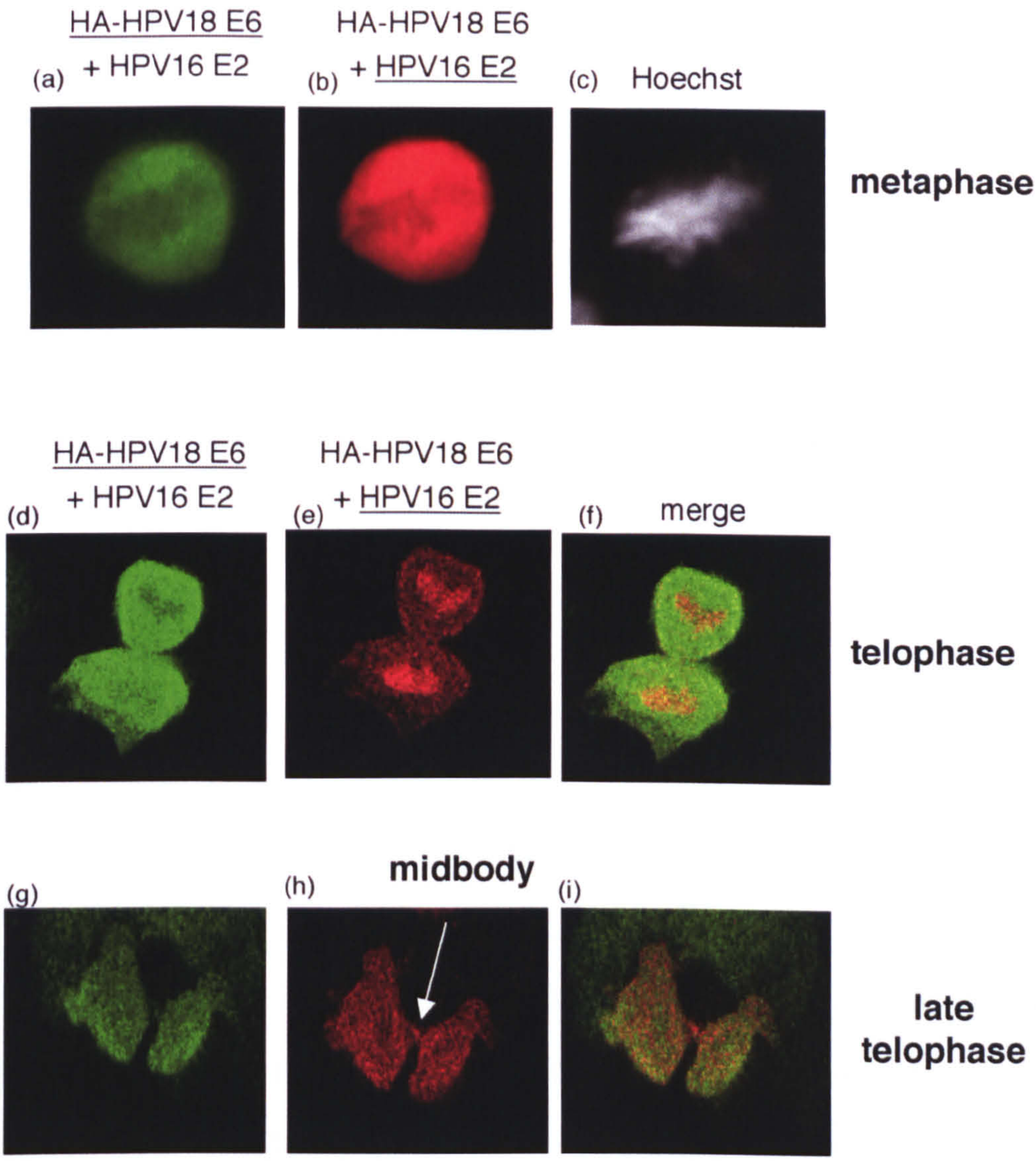


Figure 30. HPV-16 E6 does not associate with mitotic chromosomes in transiently transfected U2OS cells.

(B) E6 expression does not affect E2 association with mitotic chromosomes. U2OS cells were transiently transfected as indicated, fixed and double stained for HA-E6 (green, 12CA5, Roche) and E2 (red, anti-E2 rabbit polyclonal). The panels show staining of E2 and E6 in metaphase (a-c), telophase (d-f) and late telophase when E2 is localised in the midbody (g-i). DNA was stained with Hoechst (c).

that seen when E2 is transfected alone (Figure 30B, panels d-f). Furthermore, E2 was also found localised to the midbody during cytokineses, regardless of the presence of E6 (Figure 30B, panels g-i). In contrast, the E6 protein was excluded from condensed chromosomes and localised outside the chromatin during all stages of mitosis, either when transfected alone (Figure 30A) or when in combination with E2 (Figure 30B, panels a, d and g, respectively). These results suggest that E6 does not associate with mitotic chromosomes and does not have any affect upon E2's function during this phase of the cell cycle.

L2-E6

Some of the most striking observations from our initial screen of viral protein interactions were the associations of E6 and E7 oncoproteins with the late structural protein L2 (Figure 10, panel c and Table 1). These data were particularly surprising, since E7 and E6 are expressed from the viral early promoter (p97) only in cells of the basal layer or parabasal and intermediate cell layers of the infected epithelium (Middleton et al., 2003). In contrast expression of the viral capsid proteins (L1 and L2) is thought to follow completion of genome amplification and to occur only in the upper epithelial layers (Middleton et al., 2003). However, recent studies suggest that L2 is actually expressed prior to L1 in cells which could potentially also express E6 and E7 (Florin et al., 2002a). Unfortunately, no data are currently available about the distribution of E6, E7 or markers of viral oncogene activity, such as PCNA, in relation to L2 expression in a productive high-risk HPV infection. However, several studies have suggested that L2 serves important nonstructural functions during virus assembly and during the infection process (Day et al., 1998; Florin et al., 2002a, b; Kawana et al., 1998; Unckell et al., 1997; Yang et al., 2003). Considering that L2 accumulates in promyelocytic leukemia protein (PML) bodies (also known as PODs or ND10) and recruits L1 and E2 into these nuclear substructures where viral DNA replication and probably virus assembly takes place (Swindle et al., 1999; Day et al., 1998), we decided to investigate more closely the potential association between the viral

oncoproteins and L2.

HPV L2 induces relocalisation of E6 and E7 oncoproteins to PODs

It has been shown previously that L2 colocalises with PML in PODs (Day et al., 1998; Gornemann et al., 2002; Florin et al., 2002a). Furthermore, while BPV-1 E2 and L1 normally exhibit a diffuse nuclear localisation, in the presence of L2 there is relocalisation of E2 and L1 to PODs (Day et al., 1998; Heino et al., 2000). Interestingly, this localisation partially, or completely, overlaps with the site of HPV-11 DNA replication (Swindle et al., 1999). Therefore, we were particularly interested in determining whether the expression of the L2 capsid protein affects the distribution of the E6 and E7 proteins. To investigate this, cotransfection experiments were done with the HA-tagged HPV-18 E6 and the HPV-16 L2 expression plasmids. After 24 hrs the cells were fixed and stained for both proteins using mouse anti-HA and rabbit polyclonal anti-L2 antibodies, and the results are shown in Figure 31. As can be seen the L2 protein localises to nuclear subdomains (PODs) in a proportion of cells, as seen in previous studies (Figure 31, panel a) (Day et al., 1998; Gornemann et al., 2002; Becker et al., 2004). HPV-18 E6 alone shows a diffused pattern of expression (Figure 31, panel b), but in the presence of L2 there is a clear relocalisation of E6 to nuclear subdomains, and a punctate pattern of staining for both E6 and L2 is commonly seen in the nucleus (Figure 31, panels c-e, f-h and i-k). When HPV-16 E7 is cotransfected with HPV-16 L2 similar results are obtained. As can be seen from Figure 32, E7 alone shows diffuse nuclear staining (Figure 32, panel b), but in the presence of L2 a clear punctate pattern of staining was seen in the nuclei of these cells, indicating that L2 also relocalises HPV E7 to these nuclear subdomains (Figure 32, panels c-e and f-h). Taken together, these results suggest that L2 can induce the relocalisation of the E6 and E7 oncoproteins to distinct nuclear structures, previously identified as PODs (Day et al., 1998).

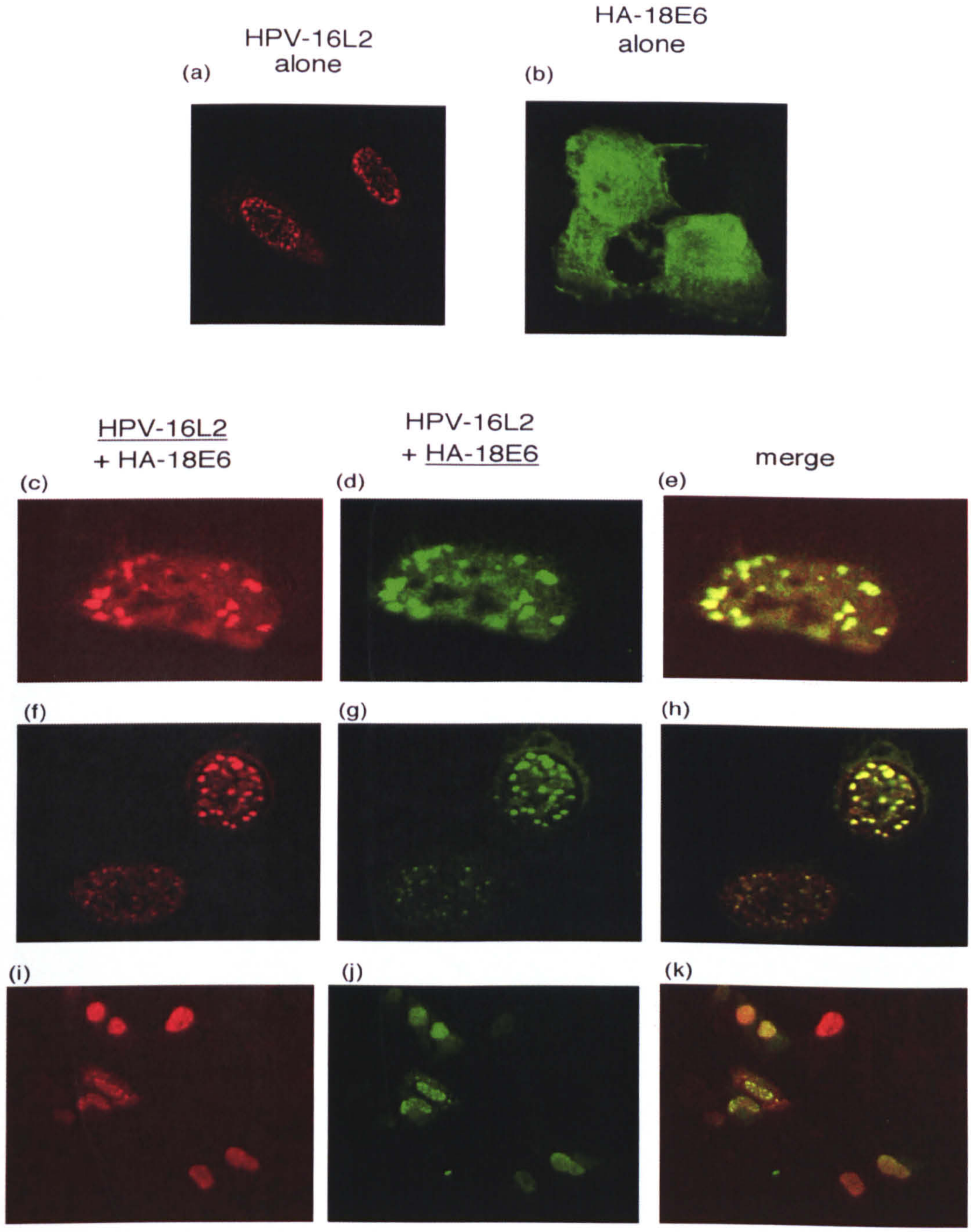


Figure 31. HPV L2 induces relocalisation of the HPV E6 oncoprotein. U2OS cells were either transfected with HPV-16 L2 (panel a) or HA-18 E6 (panel b) alone or in combination (panels c-e, f-h and i-k) and then detected by double immunofluorescence. The L2 protein was detected with the anti-L2 antiserum and rhodamine-conjugated goat anti-rabbit antibody (Molecular Probes). HA-18 E6 was detected with the anti-HA monoclonal antibody, 12CA5 (Roche) and FITC-conjugated goat anti-mouse IgG. Note the redistribution of the E6 into the L2-staining PODs (panel i) as well as speckled nuclear staining upon higher magnification (panels c and f). In each experiment a total of 30 fields were counted, and in this case out of 200 cells showing L2 as a punctate pattern, in all of them E6 was relocalised in the same nuclear compartments. Similar percentages were obtained in each of three replicate experiments.

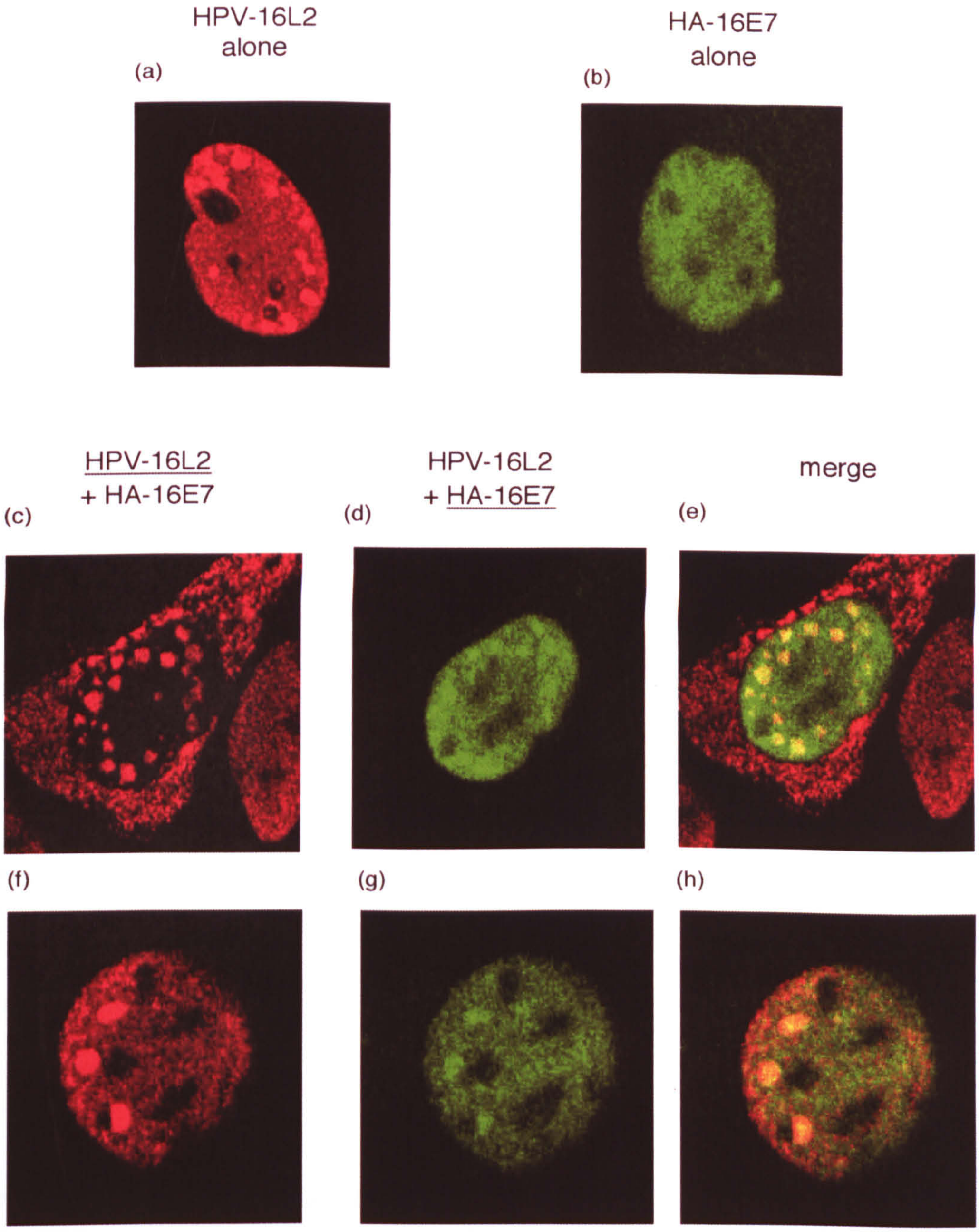


Figure 32. L2 expression affects the subcellular distribution of HPV-16 E7. U2OS cells were either transfected with HPV-16 L2 alone (panel a) and HA-16 E7 alone (panel b) or in combination (panels c-e and f-h). After 24 hrs the cells were fixed and double stained for HA-16 E7 (primary: 12CA5, Roche; secondary: fluorescein-green anti mouse, Molecular Probes) and HPV-16 L2 (primary: rabbit polyclonal anti-L2; secondary: rhodamine-red anti rabbit, Molecular Probes). In each experiment a total of 30 fields were counted, and in this case out of 120 cells showing L2 as a punctate pattern, in all of them E7 was relocalised in the same nuclear compartments. Similar percentages were obtained in each of three replicate experiments.

HPV-16 L2 is confocal with PML isoforms I, II and IV

Previous studies have shown that extensive alternative splicing of the PML gene results in at least seven different isoforms (Jensen et al., 2001). Although the function of these different isoforms is unknown, many studies have reported isoform-specific protein interactions (Fogal et al., 2000; Wu et al., 2001; Alcalay et al., 1998), suggesting that not all forms are functionally equivalent. Therefore, prior to further investigating the L2 relocalisation of E6, we were particularly interested in determining whether the HPV-16 L2 protein targets any specific subsets of PML isoforms. To do this, we made use of a panel of Flag-tagged PML isoforms that are depicted schematically in Figure 33A, and transfected these into U2OS cells. After 24 hrs the cells were fixed and stained and, as can be seen from Figure 33B, overexpression of these different isoforms gives rise to punctate nuclear staining, with some variation in the size, shape and number of dots depending on the particular isoform. The same experiment was then repeated including HPV-16 L2 and the results are shown in Figure 34. As can be seen, HPV-16 L2 shows a very high degree of confocality with PML isoforms I, II and IV. In contrast, there is no significant degree of colocalisation with PML isoforms III, V and VI. Taken together, these results demonstrate that the localisation of L2 to PODs is highly specific in that only a subset of these structures is being targeted.

Reorganization of PODs induced by HPV-16 L2

Since many DNA viruses have been reported to alter POD composition through diverse pathways, we were interested in the effects that HPV-16 L2 might have upon POD composition. This was especially important in the light of studies showing that HPV-33 L2 accumulation in PODs resulted in the reorganisation of these domains, with Sp100 being lost and the transcriptional repressor Daxx being recruited (Florin et al., 2002b). To investigate this we expressed HPV-16 L2 in U2OS cells and analysed the effect upon the subcellular distribution of two known POD-associated proteins, PML and SUMO-1. The results for PML are shown in Figure 35 and the results for SUMO-1 in Figure 36. As can be seen, a surprising redistribution

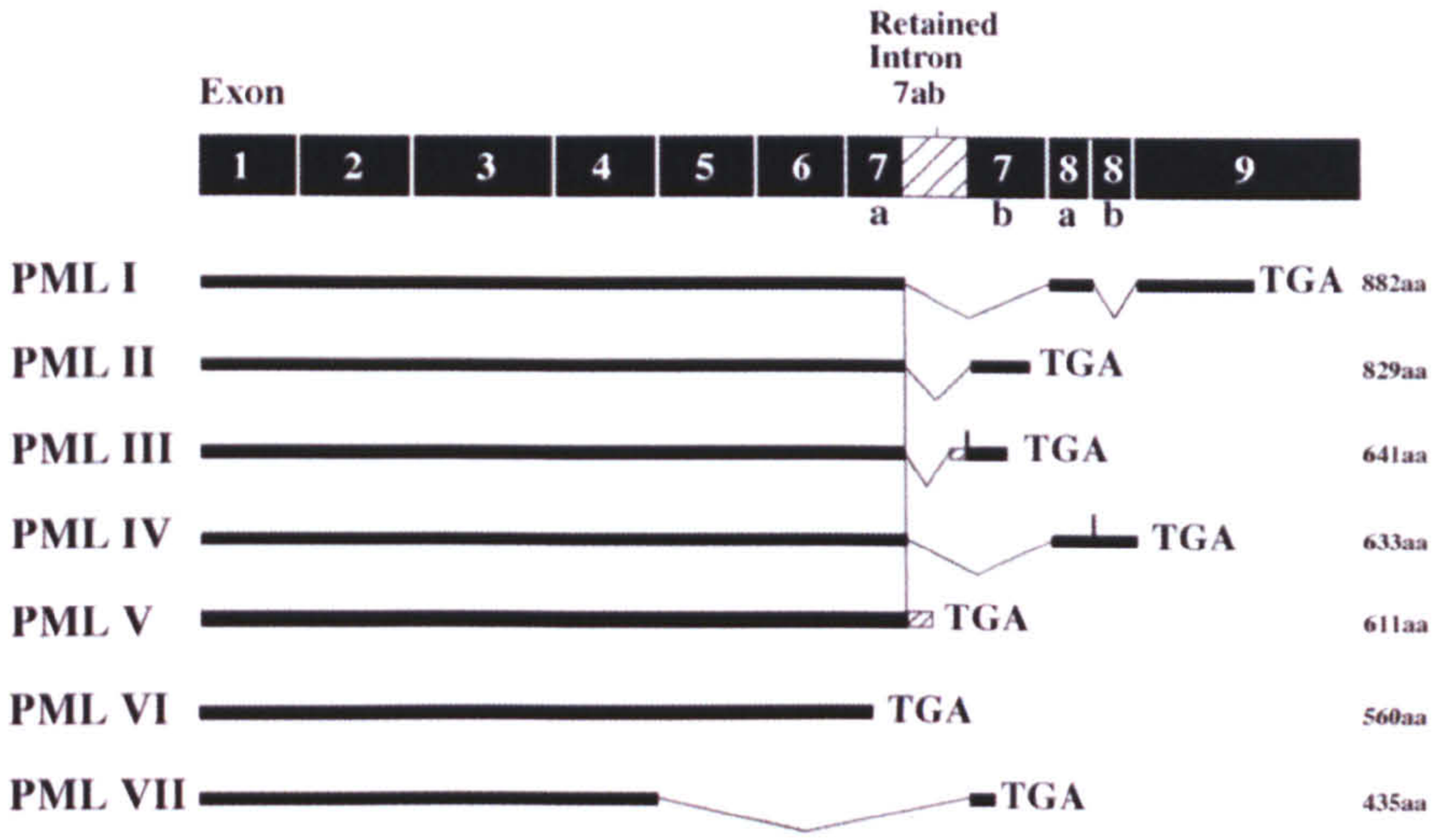
of PML occurred in cells showing a punctate nuclear pattern of L2 staining when compared with non-transfected cells (compare Figure 35, panel a with panels b-d and e-g). Interestingly, in other studies the PML distribution appeared to be unaffected by the expression of the BPV-1 L2 or HPV-33 L2 protein (Day et al., 1998; Florin et al., 2002b), suggesting that this is a specific feature of HPV-16 L2. In addition, the cellular distribution of SUMO-1 was similarly affected (Figure 36). In non-transfected cells, SUMO-1 showed a nuclear pattern that varied from a diffuse to a slightly speckled arrangement (Figure 36, panel a). This pattern was dramatically altered when the L2 was expressed and showed a strong concentration at the PODs, where it also colocalised with L2 (Figure 36, panels b-d). In contrast, in cells where L2 staining was diffuse there was no apparent change in the SUMO-1 distribution (Figure 36, panels e-g). These results suggest that the L2 minor capsid protein of HPV-16 can induce major changes in POD organisation.

Having shown that L2 can relocalise HPV-18 E6 and SUMO-1 independently into L2-staining PODs (Figures 31 and 36), we next wanted to assess the effects of coexpression of E6 and L2 upon the distribution of SUMO-1. U2OS cells were transfected with the HA-tagged HPV-18 E6 expression construct alone or in combination with HPV-16 L2. After 24 hrs the cells were fixed and costained for HA-E6 and SUMO-1. In this assay, the HPV-16 L2 protein could not be visualised due to the unavailability of a triple staining system, however, it is clear that L2 expression shifts both HPV-18 E6 and SUMO-1 into punctate regions (Figure 37, panels a-c) similar to those observed with the anti-L2 staining shown in Figure 31, panel a. In contrast, HPV-18 E6 alone showed a diffused pattern of expression and had no effect on SUMO-1 distribution (Figure 37, panels d-f). This indicates that the L2 protein can induce redistribution of SUMO-1 and HPV-18 E6 into PODs simultaneously.

L2-recruited Daxx is degraded by E6

Since previous studies had shown that Daxx was recruited into PODs by HPV-33 L2 (Florin et al., 2002b, Becker et al., 2003), it was obviously of interest to investigate whether HPV-16 L2

A.



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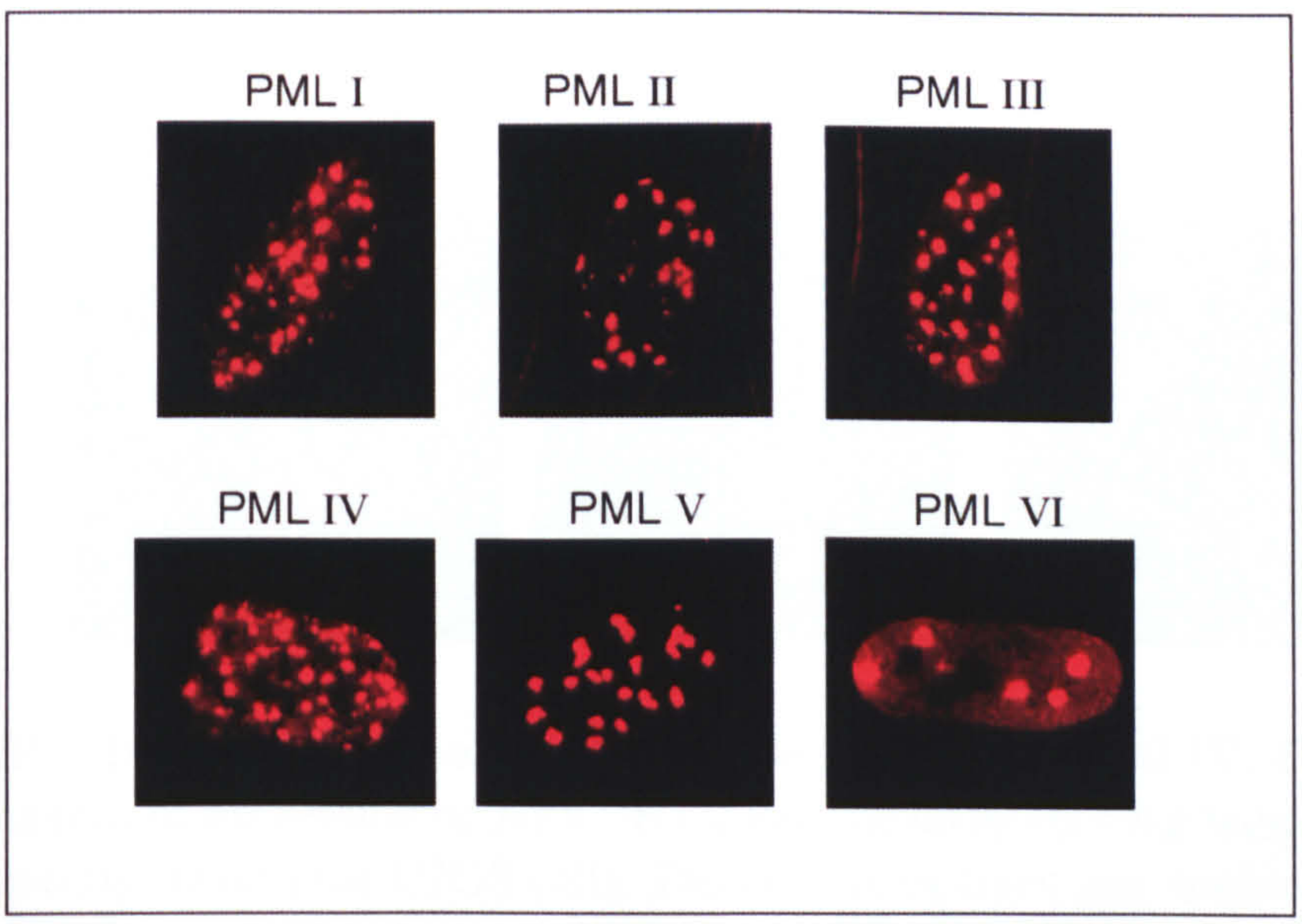


Figure 33. (A) A schematic representation of the different PML constructs used in the study. The PML isoforms are depicted as the alternatively spliced variant mRNAs that were cloned downstream of the Flag tag coding sequence (Modified from Bischof et al., 2002). (B) Localisation of the different Flag-tagged PML isoforms in transiently transfected U2OS cells. The cells were fixed and the PML detected using an anti-Flag monoclonal antibody (M2, Sigma).

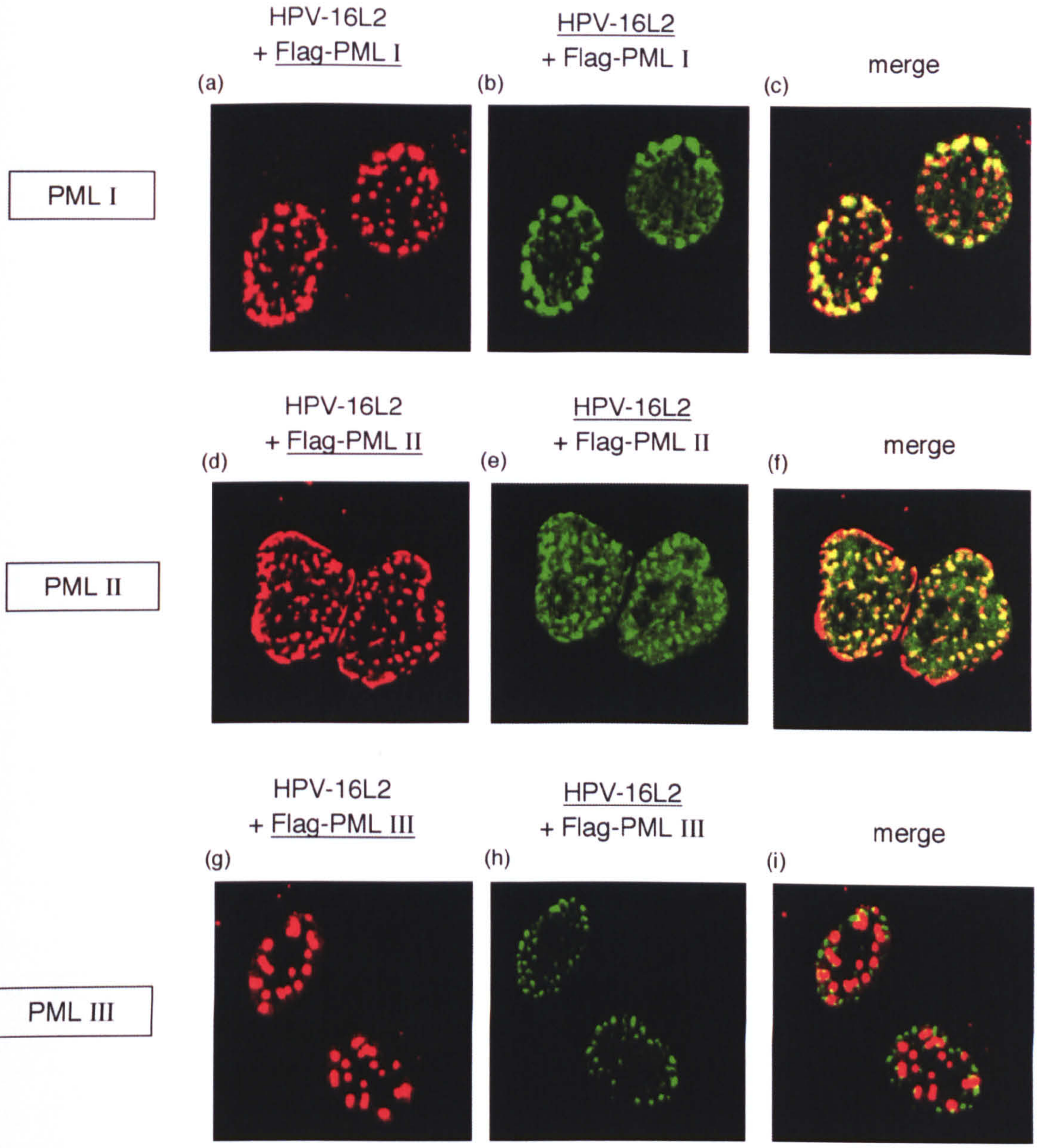


Figure 34. HPV-16 L2 colocalises with PML isoforms I, II and IV. Confocal analysis of the pattern of expression of HPV-16 L2 and the different Flag-tagged PML isoforms in transiently transfected U2OS cells. The cells were fixed and double stained for L2 (primary: rabbit polyclonal anti-L2; secondary: fluorescein-green anti rabbit, Molecular Probes) and Flag-PML (primary: M2, Sigma; secondary: rhodamine-red anti mouse, Molecular Probes). The panels show PML alone (red), HPV-16 L2 alone (green) and the merged image. The PML isoforms analysed are as follows: isoform I (panels a-c), isoform II (panels d-f), isoform III (panels g-i).

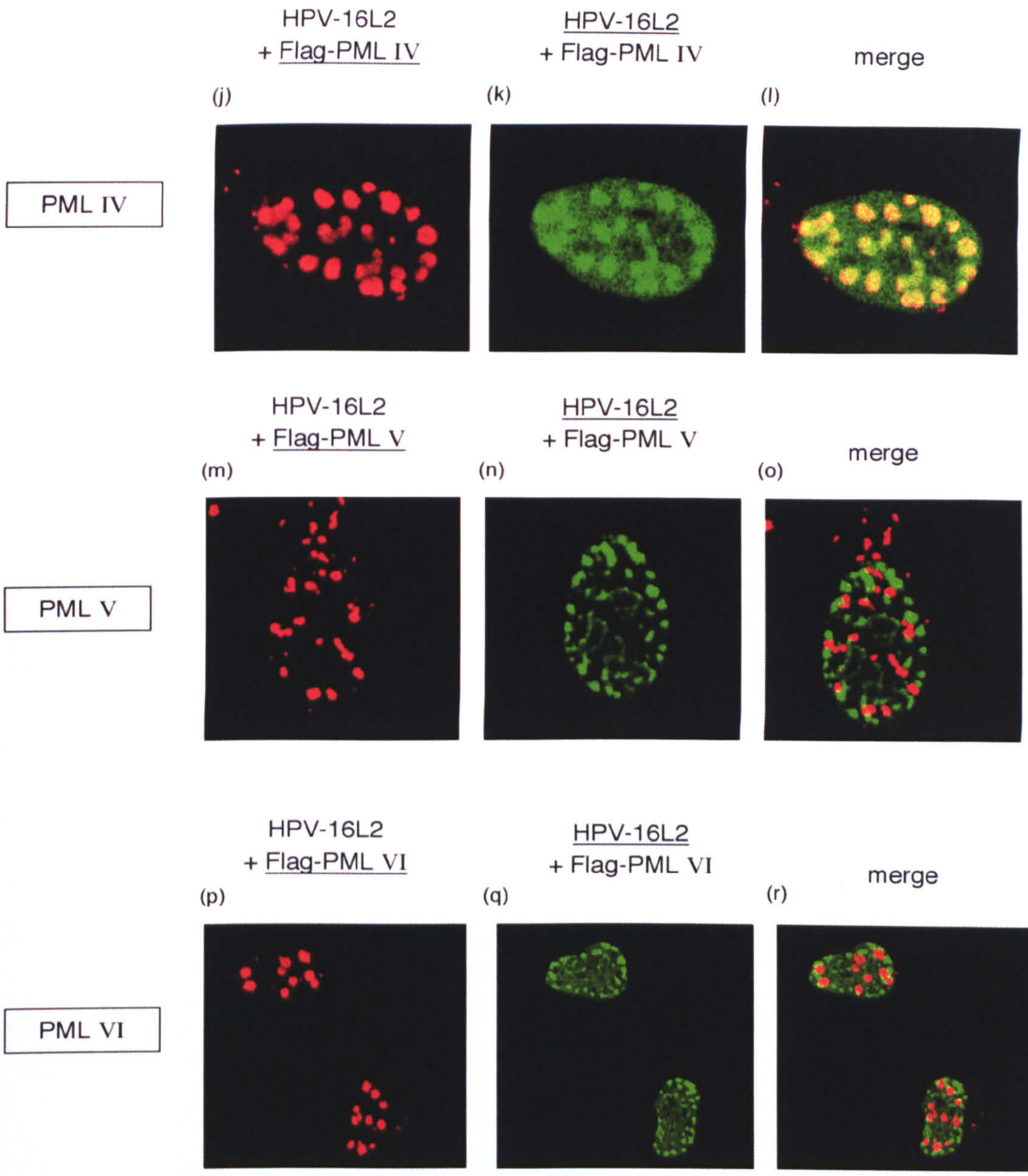


Figure 34. HPV-16 L2 colocalises with PML isoforms I, II and IV. Confocal analysis of the pattern of expression of HPV-16 L2 and the different Flag-tagged PML isoforms in transiently transfected U2OS cells. The cells were fixed and double stained for L2 (primary: rabbit polyclonal anti-L2; secondary: fluorescein-green anti rabbit, Molecular Probes) and Flag-PML (primary: M2, Sigma; secondary: rhodamine-red anti mouse, Molecular Probes). The panels show PML alone (red), HPV-16 L2 alone (green) and the merged image. The PML isoforms analysed are as follows: isoform IV (panels j-l), isoform V (panels m-o) and isoform VI (panels p-r). In each experiment a total of 20 fields were counted, and in this case out of 200 cells showing L2 as a punctate pattern, all of them showed the colocalisation with PML isoforms I, II or IV.

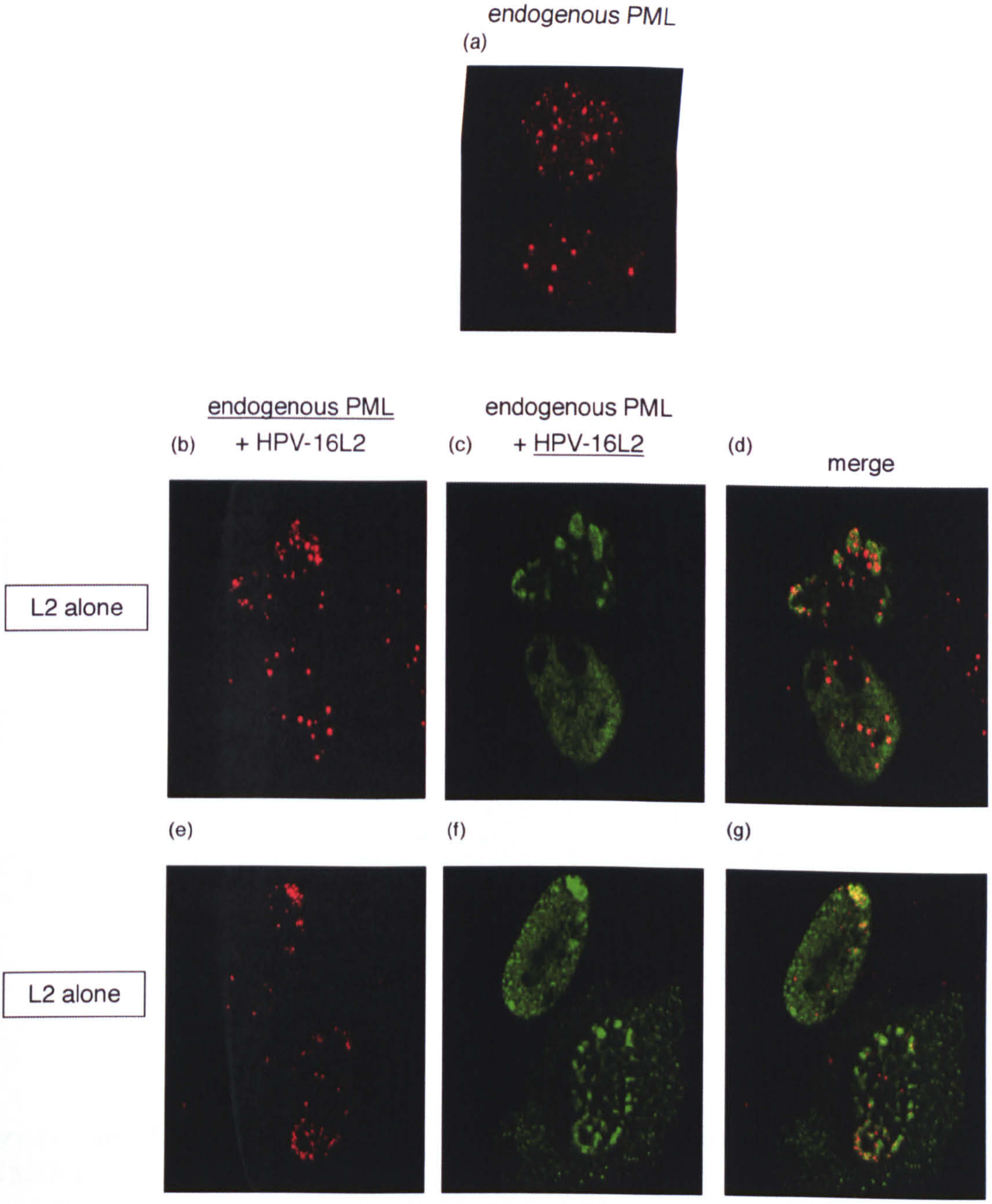


Figure 35. HPV-16 L2 affects the cellular distribution of endogenous PML. Confocal analysis of the pattern of expression of endogenous PML in U2OS cells either non-transfected (panel a) or transiently transfected with HPV-16 L2 (panels b-d and e-g). The cells were fixed and double stained for PML (primary: PG-M3, Santa Cruz; secondary: rhodamine-red anti mouse, Molecular Probes), and L2 (primary: anti-L2 serum; secondary: fluorescein-green anti rabbit). Note that in panels b-d and e-g the PML was concentrated in L2 containing nuclear speckles and was observed in all PML and L2 positive staining cells (number assayed=200).

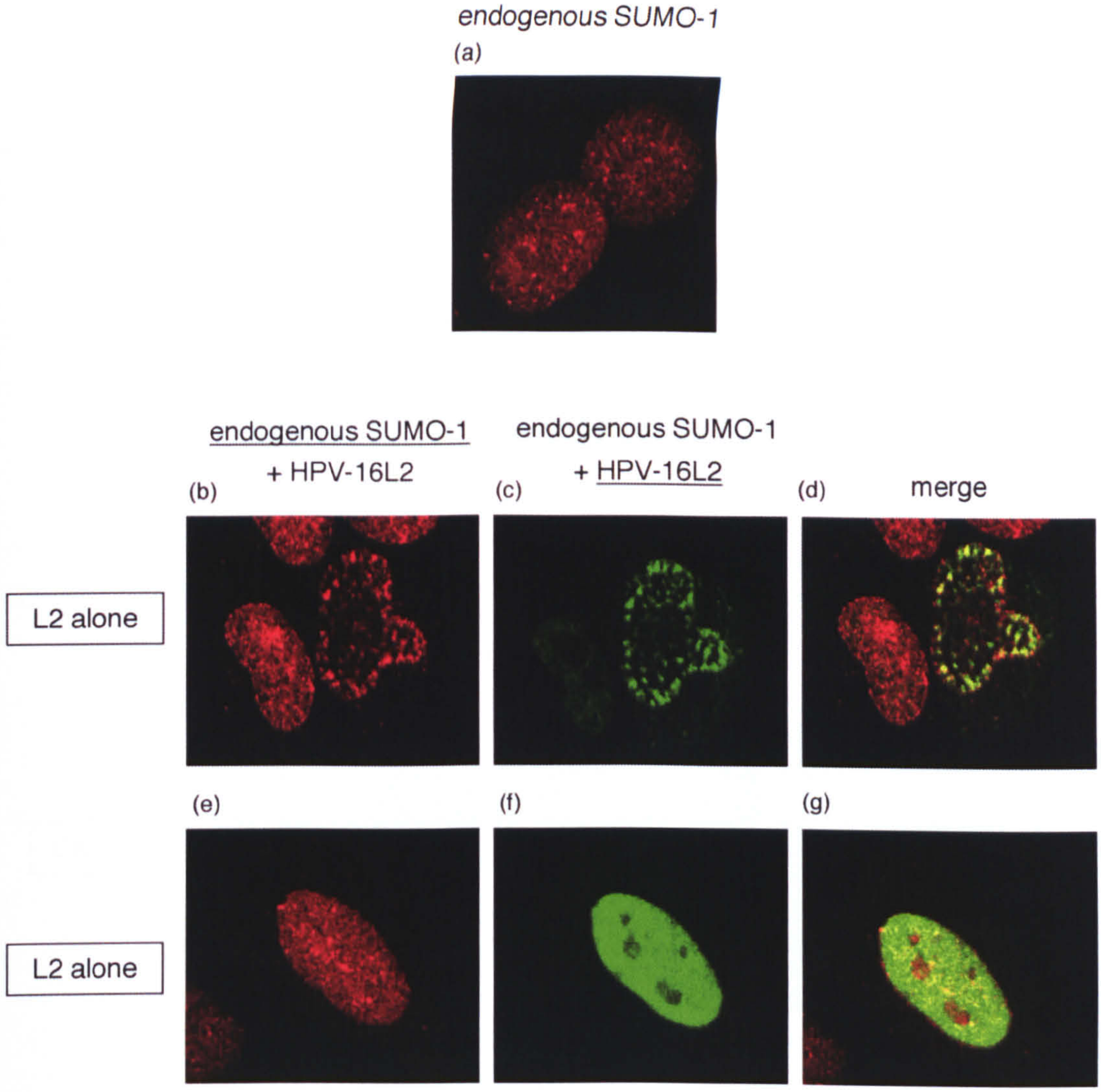


Figure 36. HPV-16 L2 affects the cellular distribution of endogenous SUMO-1.

Confocal analysis of pattern of expression of SUMO-1 and HPV-16 L2 in transiently transfected U2OS cells. The cells were fixed and double stained for SUMO-1 (red, GMP1, Zymed) and L2 (green, rabbit polyclonal anti-L2). The panels show SUMO-1 alone (red), L2 alone (green) and the merged image. Compare the cellular distribution of SUMO-1 in non-transfected cell (panel a), with the distribution of SUMO-1 when L2 is transfected (panels b-d). The SUMO-1 was concentrated in L2 containing nuclear speckles and was observed in all SUMO-1 and L2 positive staining cells (number assayed=200).

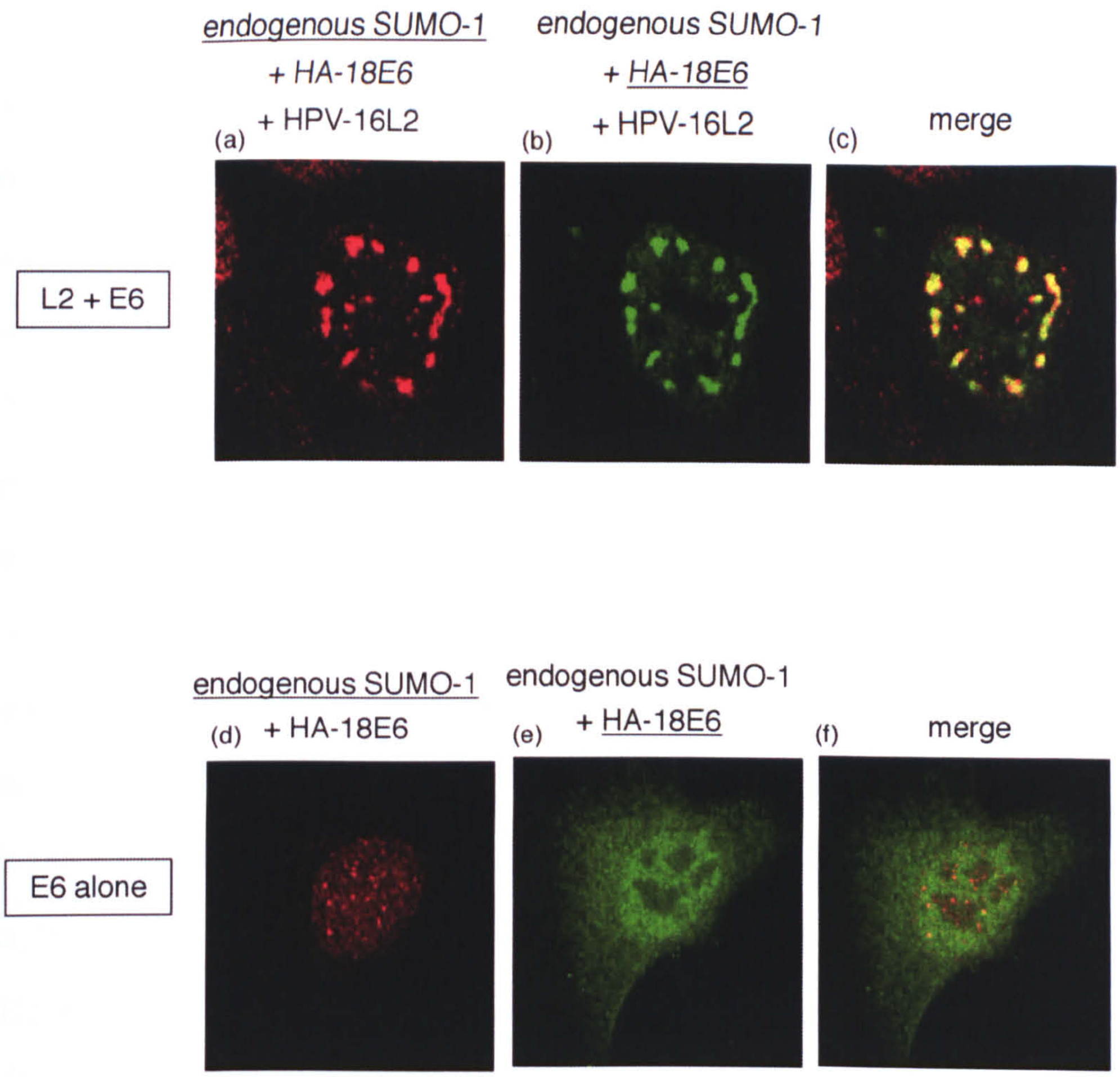


Figure 37. L2-containing nuclear bodies also contain E6 and SUMO-1. U2OS cells were transfected with a HPV-18 E6 expression plasmid alone (panels d-f) or in combination with HPV-16 L2 (panels a-c). Cells were fixed and stained with anti-HA (Y-11, Santa Cruz) and anti-SUMO-1 antibodies (GMP1, Zymed). Note that in panels a-c the cotransfected L2 is not detectable owing to a lack of triple staining technology.

could act similarly. As can be seen from Figure 38 (panels a-c) Daxx resembled the punctate staining pattern similar to that of L2. It should be noted here that, because of the lack of the suitable antibodies for simultaneous detection of L2 and Daxx, we cotransfected HPV-16 L2 together with a GFP expression plasmid to mark transfected cells and looked for Daxx staining in those cells. To test whether the L2-mediated accumulation of E6 into PODs affects Daxx, U2OS cells were cotransfected with HA-tagged HPV-18 E6 and HPV-16 L2 expression plasmids and costained for HA-E6 and endogenous Daxx proteins. Surprisingly, no accumulation of Daxx protein was observed when the L2 and E6 were coexpressed, as can be seen in Figure 38 (panels d-f and inset g-i). This result raises two intriguing possibilities: E6 may cause the dispersal of the Daxx protein throughout the nucleoplasm, or, alternatively, may induce the degradation of Daxx. Since PODs are sites of proteolytic degradation (Everett et al., 1997) and HPV-18 E6 is also intimately linked to the proteasome pathway (Mantovani & Banks, 2001), we decided to investigate whether Daxx was indeed being degraded by E6. To do this, U2OS cells were transfected with HA-tagged HPV-18 E6 and HPV-16 L2 and, after 24 hrs the cells were treated with the proteasome inhibitors N-acetyl-leu-leu-Norleucinal (LLnL) or N-CBZ-leu-leu-leu-al (CBZ) for 3 hrs. The cells were then fixed and double-stained for HA-E6 and Daxx protein and the result are shown in Figure 39. As can be seen, treatment of cells with either LLnL (Figure 39, panels a-c and d-f) or CBZ (Figure 39, panels g-i) gives rise to a dramatic increase in Daxx levels at PODs, suggesting that Daxx is indeed degraded when E6 is recruited to PODs by L2. In contrast, Daxx distribution was not perturbed in the presence of E6 alone (Figure 40, panels b-d), and was similarly unaffected by the addition of the proteasome inhibitor LLnL (Figure 40, panels f-h). Since it was previously shown that the distribution of HPV-33 L2 changes after the treatment with proteasome inhibitors (Florin et al., 2002a), we next tested if the distribution of HPV-16 L2 is also affected by proteasome inhibitors and, as can be seen in Figure 41 no significant change in the HPV-16 L2 distribution was observed in cells treated with either LLnL or CBZ (Figure 41, compare all three panels). Taken together, these results suggest that E6 induces a proteolytic degradation of Daxx when it is relocalised into

PODs by L2.

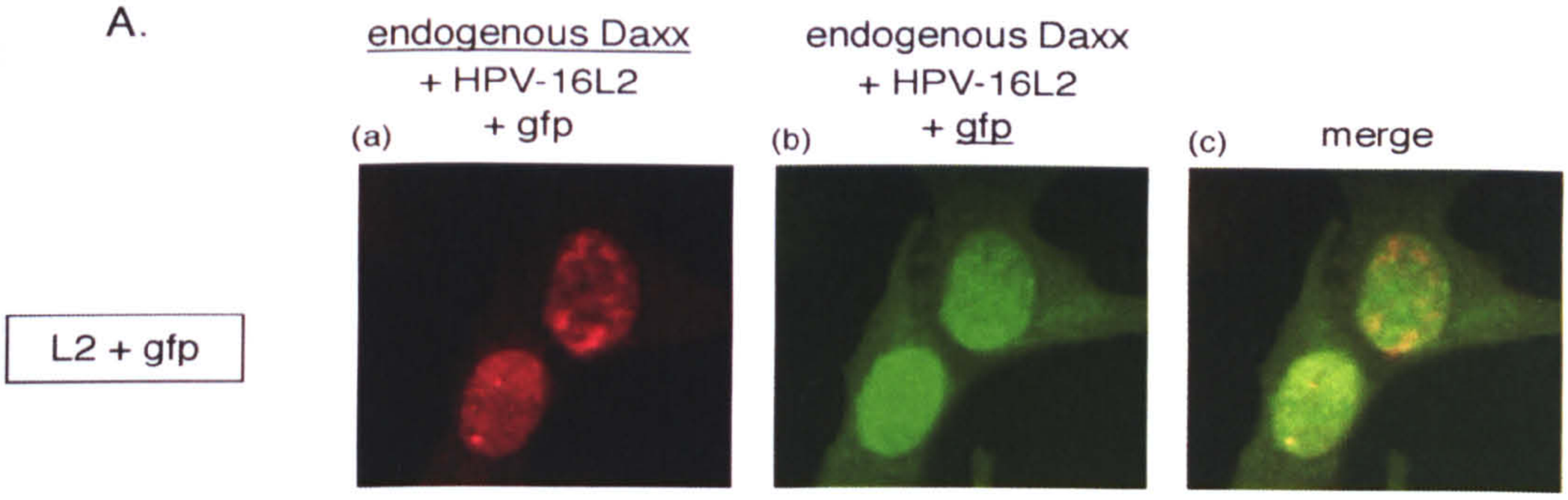


Figure 38. The effect of L2/E6 accumulation in PODs upon Daxx protein. (A) L2-containing PODs are enriched in Daxx. GFP was transiently transfected together with HPV-16 L2 and after 24 hrs the cells were fixed and stained with rabbit polyclonal anti-Daxx antibody (red, M-112, Santa Cruz) (panels a-c). Note that GFP was used as a marker of transfection, due to a lack of the monoclonal anti-L2 antibody. (B) E6 causes the dispersion of Daxx in L2-staining PODs. U2OS cells were transiently transfected with the HPV-18 E6 in combination with HPV-16 L2. After 24 hrs the cells were fixed and double stained for HA-E6 (green, 12CA5, Roche) and Daxx (red, M-112, Santa Cruz). The panels show Daxx alone (red), HA-18E6 alone (green) and the merged image. Note the loss of Daxx in E6-enriched PODs in the presence of L2 (panels d-f and inset e expanded to g-i).

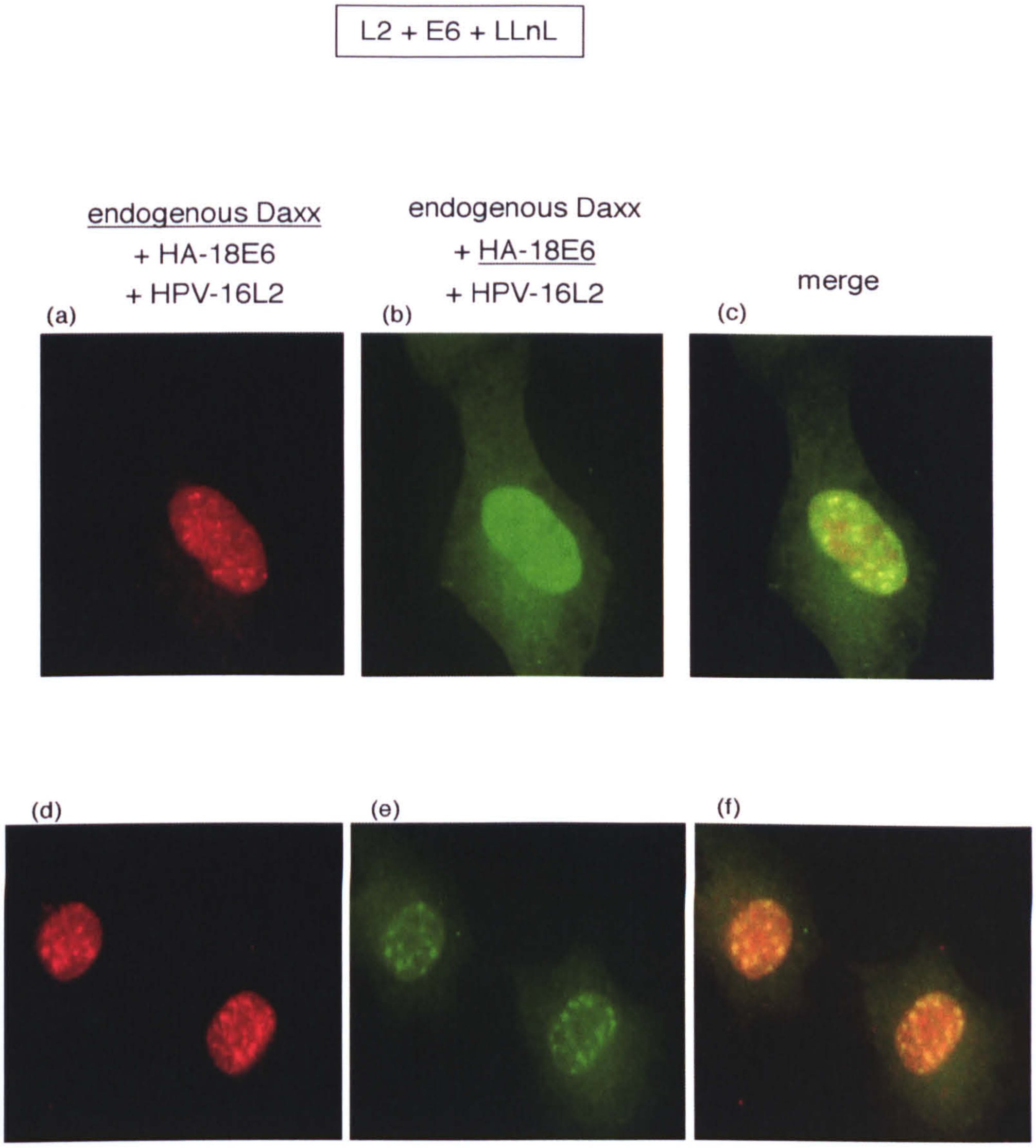


Figure 39. The HPV-18 E6 protein targets Daxx for proteasome mediated degradation in L2-staining PODs.

U2OS cells were transiently transfected with the HPV-18 E6 in combination with HPV-16 L2, fixed and stained as described in Figure 38B. The proteasome inhibitors N-acetyl-leu-leu-norleucinal (LLnL) were added 3 hours prior to immunostaining to determine whether the Daxx was being subjected to proteasome mediated degradation by E6 (panels a-c and d-f). Note the rescue of Daxx by addition of LLnL proteasome inhibitor (panels a, and d) in the presence of L2/E6 and compare with panel d in Figure 38B, which shows a clear reduction in the protein levels of Daxx in the presence of E6/L2.

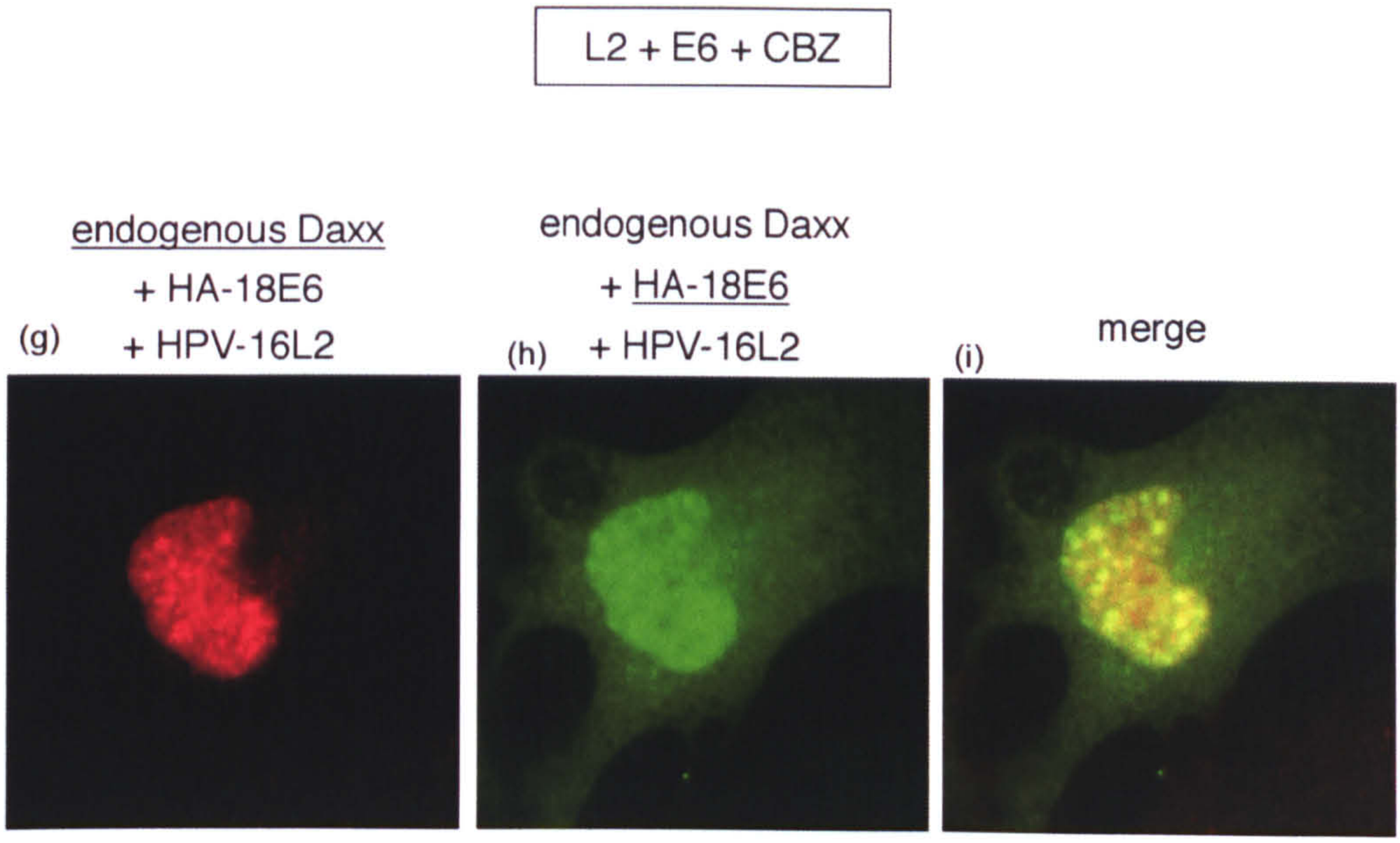


Figure 39. The HPV-18 E6 protein targets Daxx for proteasome mediated degradation in L2-staining PODs.

U2OS cells were transiently transfected with the HPV-18 E6 in combination with HPV-16 L2, fixed and stained as described in Figure 38B. The proteasome inhibitors N-CBZ-leu-leu-leu-al (CBZ) were added 3 hours prior to immunostaining to determine whether the Daxx was being subjected to proteasome mediated degradation by E6 (panels g-i). Note the rescue of Daxx by addition of CBZ proteasome inhibitors (panel g) in the presence of L2/E6 and compare with panel d in Figure 38B, which shows a clear reduction in the protein levels of Daxx in the presence of E6/L2.

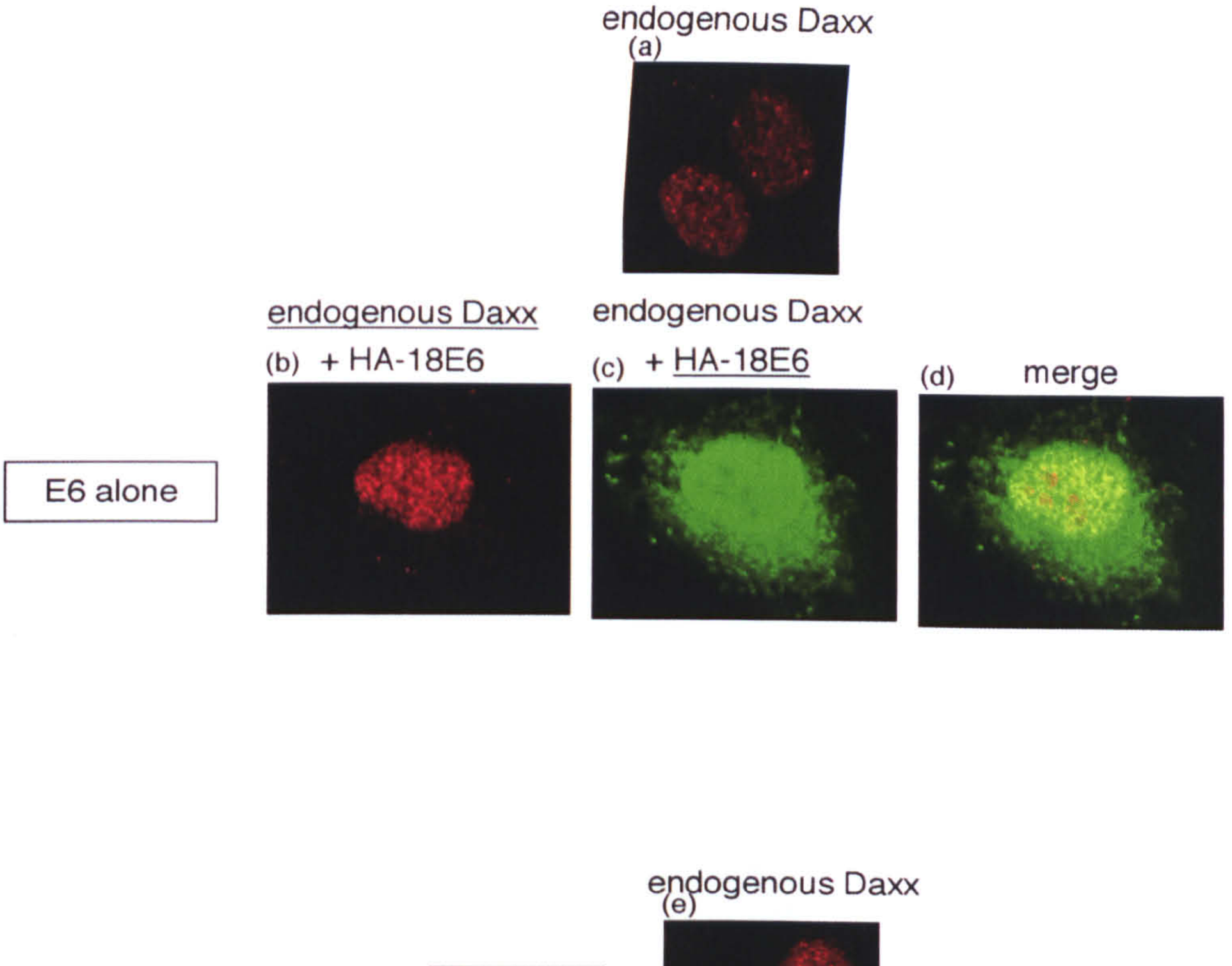


Figure 40. HPV-18 E6 does not affect Daxx levels if not relocalised by L2. U2OS cells were transiently transfected with HA-18 E6, fixed as described previously and double stained using an anti-HA mouse antibody (green, 12CA5, Roche) for HA-18 E6 and an anti-Daxx antibody (red, M-112, Santa Cruz) for endogenous Daxx. Panels b-d show double staining for E6 and endogenous Daxx in untreated cells and panels f-h for E6 and endogenous Daxx in U2OS cells treated for 3 hours prior to fixing with proteasome inhibitor LLnL. Panels a and e show the staining of endogenous Daxx in cells untreated (panel a) or treated (panel e) with proteasome inhibitor LLnL.

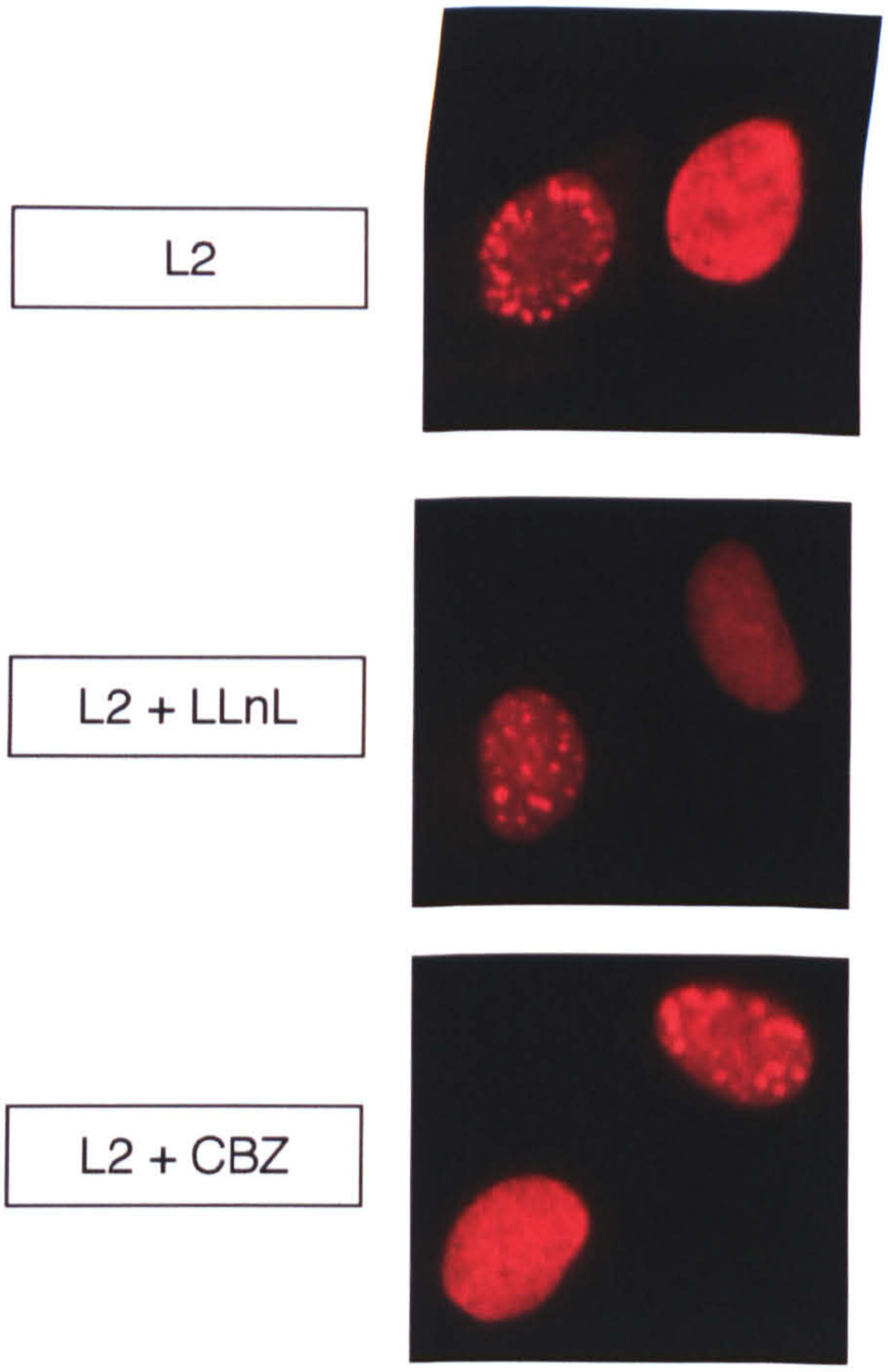


Figure 41. Effect of proteasome inhibitors, LLnL and CBZ on localisation of HPV-16 L2 in transiently transfected U2OS cells.

The cells were treated with either N-acetyl-leu-leu-norleucinal (LLnL) or N-CBZ-leu-leu-leu-al (CBZ) for 3 hrs, then fixed and stained with anti HPV-16 L2 (anti-L2 antiserum). Note no difference in localisation of L2 in the absence (top panel) or presence of proteasome inhibitors, LLnL and CBZ (middle and bottom panels, respectively).

Discussion

Papillomavirus-induced lesions arise from infection of a basal keratinocyte which subsequently proliferates as a result of viral early gene expression. The expanded population of infected cells serves as a reservoir in which the viral genome is maintained at low level. The late stages of the virus life cycle, which ultimately lead to the production of infectious particles, begin only when the infected cell migrates into the upper differentiating layers of the epidermis. Although the temporal pattern of events which trigger the late stages of the viral life cycle are not known, the regulation of viral promoters by cellular and viral factors is of critical importance. The HPV types that commonly cause lesions in the general population contain at least four promoters (Ozbun & Meyers, 1998) including the viral early promoter which lies in front of the E6 ORF, and a differentiation-dependent promoter which is contained within the E7 ORF. Although some confusion exists as to whether expression from the early promoter is upregulated (Bohm et al., 1993; Cheng et al., 1995; Chow et al., 1987; Durst et al., 1992; Stoler et al., 1989) or maintained at a steady-state level during terminal differentiation (Hummel et al., 1992, 1995; Choo et al., 1994; Higgins et al., 1992; Kyo et al., 1997), it is apparent that transcripts encoding E6 and E7 are present both in the differentiated and basal cell layers, and that these proteins may have roles other than in priming the cell for viral DNA replication. In contrast, the differentiation-dependent promoter is massively upregulated in the parabasal layers of infected epithelium, allowing high level expression of the E4 major late protein and the synthesis of transcripts encoding L1 and L2. The activity of this promoter appears to depend on an increase in viral DNA copy number (Frattini et al., 1996), although it has not yet been established if promoter activation is a prerequisite for genome amplification, or if the increase in template number in itself leads to higher levels of late gene transcription, perhaps as a result of changes in chromatin structure brought on by replication (Klumpp et al., 1997). If this is the case, then the early promoter may be downregulated during differentiation to maintain steady state levels of E6 and E7, and this would in fact be expected given the increased expression of E2 necessary

for genome amplification. High level synthesis of the E2 protein inhibits the expression from the viral early promoter (Steger & Corbach, 1997) and is necessary to efficiently recruit the E1 helicase to the viral origin of replication to allow replication to begin. Thus, a picture is emerging in which changes that occur during the initial stages of differentiation lead to activation of the differentiation-dependent promoter and expression of viral gene products necessary for DNA replication. However, there are still many interesting questions to be answered in order to understand completely the biology of the papillomavirus life cycle. The mechanism by which the switch from early to late promoter usage is mediated has not been elucidated, and the role of factors that regulate the differentiation-dependent promoter has not been characterised. Furthermore, little is known about the mechanisms that control the episomal maintenance of HPV genomes in basal cells and the virion assembly in the upper layers. To identify and understand these aspects of the viral life cycle, a lot of attention has been focussed on the interactions of viral proteins and viral DNA with other cellular proteins. However, very little attention has been placed on the possibility that the viral proteins can interact with each other and as a consequence provide additional levels of regulation to the viral life cycle. It is clear that all of the viral proteins are likely to have roles at multiple stages during a productive infection, and research in this area is not yet well advanced. Furthermore, based on the work presented here it would now appear that many of the viral proteins are capable of interacting with each other. Thus we show in a series of assays performed *in vitro* that E2 not only interacts with L2 and L1, but also has the ability to associate with E4, E6 and E7. Some of these interactions, such as E1 with E2 (reviewed in Liu & Melendy, 2002), L1 with L2 (reviewed in Howley & Lowy, 2001), and E2 with L2 (Heino et al., 2000; Okoye et al., 2005) have been described previously; the other associations represent new possible roles for the PV proteins in the viral life cycle. The most striking result from our screen however, was that the E2 and L2 proteins potentially interact with all the PV encoded proteins that were tested. It should be noted at this point that we used the N-terminal portion of a cellular protein, NT-Dlg as a negative control, and confirmed the specific nature of these associations *in vitro*. Apart from E6-E2 and

L2-E6 interactions, which are discussed below it still remains to be verified whether other new potential interactions occur *in vivo* and, if they do, what are the biological consequences. The E2 protein is expressed early in the viral life cycle and is a major regulator of viral gene expression and DNA replication. The L2 protein, on the other hand, is a structural minor capsid protein expressed late in the viral life cycle, after the differentiation of the infected keratinocyte has occured. Besides its role in capsid formation, recent studies indicate additional functions for this protein, one of them being a role in assembly and maturation of the virus (Day et al., 1998). The significance of why both E2 and L2 interact with other PV encoded proteins is currently unknown, however, it is tempting to speculate that may be through these interactions that the virus controls its life cycle.

Previous studies have shown that when HPV proteins that are known to associate *in vitro* are coexpressed, the distribution in the cell of one of them often changes, as exemplified by the coexpression of E1 with E2 (Swindle et al., 1999), L2 with E2 (Day et al., 1998), L1 with L2 (Florin et al., 2002a) and E2 with E4 (John Doorbar personal communication). In the case of E6 and E2, the first set of evidence supporting the association between E2 and E6 *in vivo* came from our immunofluorescence studies. Previous work had shown that the E2 protein has a predominantly nuclear localisation (Day et al., 1998), whilst the high-risk HPV E6 proteins localise throughout the cell (Sherman & Schlegel, 1996; Guccione et. al., 2002). However, when the two proteins were coexpressed, there was a dramatic increase in E6 levels within the nucleus. Moreover, the two proteins colocalised at splicing factor compartments (SFCs), irregular but discrete domains most frequently visualised with an antibody directed against the spliceosome assembly factor SC-35 (Fu & Maniatis, 1990). These domains were originally defined on the basis of the presence of high concentrations of pre-mRNA splicing factors (Spector, 1990; Spector et al., 1991). In addition to pre-mRNA splicing factors and snRNAs, SFCs also contain transcription factors (Larsson et al., 1995; Mortillaro et al., 1996; Zeng et al., 1997), 3'-processing factors (Krause et al., 1994; Schul et al., 1998) and ribosomal proteins (Mintz et al., 1999). Several functions have been proposed for this peculiar organisation of

splicing factors. One possibility is that SFCs are a means to control the concentration of splicing factors in the nucleoplasm and thus the sites of transcription and splicing. A second possibility is a role for SFCs in recycling, or reactivation, of splicing factors. Third could be the control of the relative ratios of multiple splicing factors in the nucleoplasm to modulate alternative splice-site selection. And last is that the SFCs represent the sites of assembly of protein complexes containing components of the transcription and RNA-processing machinery, thus forming "transcriptosomes" or "RNA processosomes" to promote the coupling of transcription and RNA processing (Misteli, 2000). Evidence now suggests that most, if not all, splicing factor domains associate with numerous genes and contain mRNAs derived from them. The placement of specific genes near these concentrated domains of RNA metabolic factors suggests a specific nuclear organisation to the gene relative to the domain which would facilitate the rapid reassembly and use of large RNA metabolic complexes for highly expressed and complex genes (Misteli, 2000). The reason for the colocalisation of E2 and E6 in these domains remains to be determined, nonetheless these data raise the intriguing possibility of a new role for the two proteins in the control of RNA splicing. It is interesting to note that the early to late switch in PV gene expression is regulated, at least partially, at the level of splicing (Baker, 1997). However, the mechanism by which the posttranscriptional block on L1/L2 expression is relieved in the upper spinous and granular layers is uncertain, suggesting a possible role for the E2/E6 complex. Our observation is even more intriguing in the light of studies with adenovirus, since it has been suggested that the E1B 58K protein and the 34K E4orf6 protein form a complex which modulates viral mRNA metabolism (Sarnow et al., 1984; Ornelles & Shenk, 1991; Dix & Leppard, 1993), with the E1B 58K acting as the RNA binding component of the complex (Horridge & Leppard, 1998). Whether HPV E6 can interact with RNA or whether the E6/E2 complex has a similar role to the adenoviral E1B/E4orf6 complex in the viral life cycle has yet to be established.

An additional set of immunofluorescence data showing that E2 and E6 indeed affect each other's localisation in the cell was obtained when we used a non-splicing mutant of E6 that was

no longer capable of producing E6*I (E6 NS). This mutant, expressing only full length E6, showed a preferentially speckled nuclear pattern of E6 expression in agreement with previous observations (Guccione et al., 2004b) and, interestingly, E2 was recruited by this mutant into the same structures. These domains correspond to PODs (Guccione et al., 2004b) and are quite separate from the SC-35 containing speckles seen when E6/E6*I and E2 are coexpressed. Thus, both E2 and E6 can significantly affect each other's localisation within the cell, indicating a potential for regulating each other's activities. In the case of a mutant E6, expressing only the full length E6 protein, E2 is recruited into POD structures, whilst in the case of wild type E6 expressing both full length and E6*I, both forms of E6 are recruited to SC-35 containing nuclear speckles in the presence of E2. Based on these observations we can conclude that both E2 and E6 can radically affect each other's pattern of expression depending on the forms of E6 being expressed.

The immunofluorescence analysis provided additional interesting information, since not all the cells positive for E2 showed strong nuclear staining of E6 protein, thus suggesting that the formation of the active E2/E6 complex may depend on the status of the cell cycle. We found that the localisation of E6 is changed preferentially in the presence of E2 during S phase, based on positive BrdU staining, since in out of 1000 cells analysed in 5 different assays, an average of 50% of cells showing the nuclear retention of E6 in the presence of E2 were positive for BrdU staining characteristic of early S phase. In addition, the E6 relocalisation was also seen in an average of 50% of cells that were negative for BrdU staining. These results could have two possible explanations. Either the E2/E6 complex forms twice during the cell cycle, once in early S phase and once in another unknown phase of the cell cycle, or, alternatively, and perhaps most likely, the complex actually forms in late G1 and is maintained into early S phase. It should be emphasised that the E2-E6 colocalisation seen in early S phase is highly specific since it was not seen in cells in mid and late S phase nor during mitosis.

We also investigated the effects of E2 and E6 coexpression upon their respective levels of expression, and observed major differences in the pattern of expression in different cellular

pools, as well as in the ratio of E6/E6*I expression. In cytoplasmic and nuclear soluble fractions the level of E6*I protein is markedly increased in the presence of E2. In contrast, the level of full length E6 is increased in the nuclear insoluble fraction in the presence of E2. Furthermore, the level of soluble E2 does not change, but the level of E2 in the insoluble fraction is also greatly increased in the presence of E6. Since we showed that these changes were not a result of changes in the pattern of mRNA expression, this suggests that E2 stabilizes the E6*I peptide, thereby changing the E6/E6*I ratio and that the E6/E2 interaction also results in both E2 and full length E6 being retained in the nuclear insoluble fraction of the cell, which is consistent with the results from the immunofluorescence analysis.

Taken together, the *in vivo* data strongly suggest that the E2 and E6 proteins can interact at a post-translational level. Therefore, we next performed a series of studies to investigate the nature of this association. The first important observation came from the *in vitro* studies showing that the E6 proteins from both high-risk virus types, HPV-16 and HPV-18 E6, strongly interact with the E2 from high-risk HPV-16. Interestingly, the low-risk HPV-11 E6 did not bind to the low-risk HPV-11 E2, suggesting that the E2/E6 interaction is high-risk virus specific. When the immunofluorescence studies were performed using low-risk HPV-11 E2 and E6 proteins, we observed two different patterns of expression. In a subset of cells the two proteins did not colocalise, confirming the *in vitro* data. However, we also observed colocalisation of the two proteins in a proportion of the E2 and E6 expressing cells. These observations can be explained by previous studies, which showed that HPV-11 E6 and HPV-11 E2 localise in dot-like structures identified as PODs (Guccione et al., 2002; Swindle et al., 1999), suggesting that the colocalisation which we observed is a result of the two proteins binding to PML and not to a direct interaction between HPV-11 E6 and HPV-11 E2. This might suggest therefore that low-risk HPV-11 E6 and HPV-11 E2 may perform similar functions with respect to each other as seen with high-risk E6 and E2, but that the mechanism by which it occurs is different.

In a series of far western assays, we also confirmed that HPV-16 E2 and E6 are capable of forming a direct protein-protein interaction. The site of interaction on E2 was mapped to a

region spanning amino acid residues 306-322 within the carboxy terminus of the E2 protein. Since this region lies adjacent to the DNA recognition helix we tested the possibility that E6 might affect the E2 DNA binding activity, but found no major effects, although whether E6 is complexed with E2 while it is bound to DNA remains to be determined. However, this region of E2 also contains two amino acids, residues 317 and 319, necessary for E2 dimerization (Hegde & Androphy, 1998) and it will be of interest to determine whether this aspect of E2 function is affected in the presence of E6. Using a panel of previously described E6 mutants we also mapped the site of the E2 interaction on E6 to amino acid residues 28-31, a region deleted in mutant E6 ΔM.

To confirm that E2 and E6 could also interact *in vivo*, a series of coimmunoprecipitation assays were done, and found clear evidence of an association between E2 and E6, but no interaction between E2 and the E6 ΔM mutant. As a further confirmation that the E2-E6 interaction was responsible for the alteration in the pattern of E6 expression, E2 and the E6 ΔM mutant were cotransfected and we found that E2 had no significant effect on the diffuse pattern of expression of the mutant E6 protein. Interestingly, the region encompassing the ΔM mutation is also found in E6*I which also binds to E2. Since previous studies have shown interplay between E6 and E6*I (Pim et al., 1997, Pim and Banks, 1999), it is striking that E2 can also serve to alter the ratio of expression of the two viral proteins. However, whether this is a direct consequence of E2 binding E6 or occurs indirectly through cell cycle effects remains to be determined (Guccione et al., 2004b).

Previous studies have shown that E2 can induce apoptotic cell death in HPV-positive and HPV-negative cell lines (Desaintes et al., 1997; Demeret et al., 2003; Webster et al., 2000). The involvement of p53 in E2-induced apoptosis has been controversial. Apoptosis induced by HPV-18 E2 has been shown to occur independently of p53 (Desaintes et al., 1997; Demeret et al., 2003), a result that contrasts with a report showing that the apoptotic activity of HPV-16 E2 depends on p53 (Webster et al., 2000). Nevertheless, the existence of alternative pathways of p53 activation, independent of the repression of E6 expression, have been proposed since the

BPV-1 E2 K344 mutant protein was shown to be able to induce p53 apoptotic activity, despite being defective for binding HPV DNA (Desaintes et al., 1997). Having observed the interaction between E2 and E6, we were naturally interested in investigating the effect of this interaction upon the ability of E6 to target p53 for proteolytic degradation. Our results demonstrate only weak rescue of p53 from E6-mediated degradation in the presence of E2 *in vivo*, and the data were confirmed with degradation assays performed *in vitro*.

We then proceeded to determine the effects of E2 upon the ability of E6 to direct the degradation of a different class of substrates, the PDZ domain-containing proteins. In this case, the degradation of three PDZ-containing proteins, MAGI-1, MAGI-2 and MAGI-3, in the presence of either HPV-16 or HPV-18 E6 is markedly inhibited by coexpression of the E2 protein *in vivo*. This inhibition was not observed with the HPV-18 E6 ΔM mutant, which still induces degradation of MAGI-3, but does not bind E2, confirming that the direct interaction between E6 and E2 is largely responsible for the inhibition of the E6-mediated degradation of the MAGI proteins. Surprisingly, the *in vitro* data does not show any protection of MAGI-1 from E6-mediated degradation in the presence of E2, suggesting that the E2-induced relocalisation of E6 into the nucleus is the most likely explanation for this protection. Based on these studies it is now clear that the E2-E6 interaction may act to regulate each protein's functions, and the obvious conclusion from this is that great care needs to be taken in interpreting the different activities of E2 that have been described with respect to its ectopic expression in HPV transformed cell lines.

The E2 protein is a major regulator of viral transcription and DNA replication and, obviously, it was of interest to determine whether E6 affects these functions of E2. Our data show that amplificational replication in a short-term DNA replication assay can be suppressed by wild type HPV-16 E6, HPV-18 E6 and the full length non splicing mutant E6 (HPV-18 E6 NS), as well as a mutant of E6 that is impaired for binding to p300 (HPV-16 E6 Δ123-7). However, this repression of viral DNA replication was not observed with the HPV-18 E6 ΔM mutant. Since this mutant fails to bind E2 this strongly suggests that inhibition of viral DNA replication is due

to the interaction between E2 and E6.

To investigate the effects of E6 upon E2 mediated transcriptional transactivation, we had to make use of a mutant E6 protein (Δ123-127) which fails to bind p300, since previous studies had shown interactions between E6 and p300 (Patel et al., 1999; Zimmermann et al., 1999, 2000), as well as between p300 and E2 (Lee et al., 2000a; Marcello et al., 2000). Thus, using a wild type E6 it would be impossible to determine which effects were p300-related. Surprisingly, when E6 Δ123-127 was cotransfected with E2 we obtained a modest increase in E2-dependent transactivation, suggesting that whilst E6 may downregulate the DNA replication activity of E2, it can meanwhile activate its transcriptional activity. However, we have to emphasize that all the transcriptional assays were performed using a synthetic promoter with 6 E2 binding sites together with a CMV promoter driving expression of E2. Obviously, use of natural promoters for E2 expression and transcriptional activation could result in a different outcome.

Exogenous E2 protein efficiently represses the expression of E6/E7 from integrated HPV genomes and consequently it was assumed that in the normal life cycle of HPV, the E2 protein functions as a transcriptional repressor. However, other studies using episomal HPV DNA showed that E2 does not repress the activity of the HPV promoter, rather they showed a steady increase of E6 and E7 transcripts (Crum, et al., 1989; Durst et al., 1992; Higgins et al., 1992; Stoler et al., 1989; Bechtold et al., 2003). Our data are consistent with the latter studies, since we show a minor increase in transcription in transient reporter assays, suggesting a role for E6 as a moderate coactivator in the normal life cycle, where the HPV genomes are episomal. Furthermore, recent studies showed that E6 is necessary for the long-term maintenance of HPV genomes as episomes in keratinocytes (Thomas et al., 1999a), and it is tempting to speculate that the E6-E2 protein-protein interaction could have a role in this. Further studies will elucidate this issue. Taken together, these data suggest that E6 may moderately increase E2-mediated transcription whilst it is able to block replication and an obvious question is how this effect is obtained. A resonable explanation could be the change in the cellular localisation, since we show that E2/E6 complex forms at the splicing factor compartments (SC-35 bodies) thaught to

be active sites of transcription. In contrast, PODs have been suggested as sites of DNA replication, thus E6 might block replication and activate transcription by simply relocalising the E2 from PODs to SC-35 nuclear speckles.

Many persistent DNA viruses have evolved a mechanism to faithfully segregate their genomes into daughter cells by tethering them to cellular mitotic chromosomes. For each virus, a virally encoded DNA-binding protein specifically binds to repeated sites in the viral DNA and tethers the genome to the condensed chromosomes via a protein-protein interaction. EBNA-1 (Epstein-Barr virus nuclear antigen-1) and LANA (latency-associated nuclear antigen) are the viral tethering proteins of Epstein-Barr virus (EBV) and human herpes virus 8 (HHV8), respectively (Ballestas et al., 1999; Hung et al., 2001). The E2 protein fulfills this role for papillomaviruses (Skiadopoulos & McBride, 1998; Lehman & Botchan, 1998); the dimeric DNA-binding domain interacts with specific binding motifs in the genome and the transactivation domain attaches to mitotic chromosomes through the cellular bromo-domain protein, known as Brd4 (Bastien & McBride, 2000; Ilves et al., 1999; You et al., 2004). Athough all the studies so far described have focussed on BPV-1, a requirement for mitotic association of the viral DNA in dividing cells is no doubt a general feature of all PVs, including the HPVs (Howley & Lowy, 2001). Indeed several cell lines derived from HPV-16 and HPV-31 pre-neoplastic lesions have been shown to harbor predominantly extrachromosomal viral DNA (Bedell et al., 1991; Stanley et al., 1989). Furthermore, replication assays using HPV-31 have shown roles for E2 protein and E2 binding sites during viral genome maintenance in cultured cells (Hummel et al., 1992; Stubenrauch et al., 1998, 2000). In our studies, we have shown that HPV-16 E2 protein also interacts with mitotic chromosomes, thereby supporting the hypothesis that different PVs share a common, general mechanism of viral genome segregation. However, it has been previously shown that BPV-1 E2 remains associated with mitotic chromosomes at every stage of mitosis (Bastien & McBride, 2000). In contrast, we observed HPV-16 E2 associated with mitotic chromosomes only during cytokinesis, which begins with anaphase and continues through telophase until the formation of the midbody that persists between the two daughter cells, to

which E2 was also found to localise. Taken together, these data suggest that different papillomaviruses may use E2 proteins to segregate viral genomes between the daughter cells, but that the precise mechanisms involved are probably different. The E6 protein was excluded from condensed chromosomes and localised outside the chromatin throughout mitosis, whether transfected alone or in combination with E2, suggesting that E6 does not associate with mitotic chromosomes and has no effect upon E2's function during this phase of the cell cycle.

Taken together, our data suggest new roles for E6 and E2 in the regulation of the HPV life cycle, and provide compelling evidence for how the loss of E2 expression might lead to uncontrolled activities of E6. Papillomaviruses must infect basal epithelial cells to establish a productive infection during which episomal maintenance of the viral genome in cells is essential for the establishment of infection. The E6 and E7 proteins hold the cell in cycle to allow synthesis of the cellular components required for DNA replication (Cheng et. al., 1995). We can speculate that E6 might further activate the viral early promoter by binding to E2 in this initial step (Figure 42, upper panel). However, amplificational replication must be controlled to avoid over-replication and the consequent risk of unscheduled death of basal or suprabasal cells, because the synthesis of late genes takes place only in terminally differentiated epithelial cells. It is tempting to speculate that the ability of E6 to block the papillomavirus amplificational replication is actually used by the virus to control the productive infection of basal cells (Figure 42, upper panel). Whilst E6 clearly has potential to modulate E2 function with respect to viral gene expression and HPV genome replication, it is clear that the reverse is also true with respect to E6 function in directing the degradation of some of its cellular substrates, as well as affecting the levels of E6*I expression. Thus it seems likely that while E2 levels are low E6 will have a different pattern of expression and will be able to target cytoplasmic PDZ-domain containing substrates, such as the MAGI proteins. However, as the viral life cycle progresses and E2 levels increase E6 will be relocalised to nuclear speckles and its substrate specificity will change. Obviously at this stage it is difficult to speculate on the relevance of this for viral replication, however it is clear that the implications for loss of E2 during malignant progression are much

more profound than previously thought. Thus, while overexpression of E2 may indeed suppress HPV gene expression, there are many caveats as to whether this actually occurs *in vivo*. We show here, rather, that loss of E2 will simply deregulate E6 function at the post-transcriptional level, and we thereby offer a more direct explanation as to why loss of E2 is favored during malignant progression (Figure 42, lower panel).

Having investigated the biological implications of the E2/E6 interaction, we next proceeded to investigate the intriguing interaction between E6 and L2. The exact role of the L2 protein in the virus life cycle is still unclear. While there are 360 L1 molecules present in a virion, it has been estimated that there are only 12 L2 molecules (Trus et al., 1997). There have been reports that L2 is required for the generation of infectious virions or pseudovirions and that L2, in addition to L1, interacts with a cell surface receptor (Kawana et al., 2001; Okun et al., 2001). In addition to having a role in viral entry, the L2 protein might be a key factor during assembly and maturation of the virus. When expressed in tissue culture cells, L2 can accumulate in distinct nuclear dots that have been identified as promyelocytic leukaemia oncogenic domains (PML-oncogenic domains or PODs, PML bodies, ND10) (Day et al., 1998; Florin et al., 2002a; Gornemann et al., 2002). The function of PODs has remained largely enigmatic. Given the multitude of proteins that may associate with these structures, albeit some only transiently, PODs are unlikely to serve any single specific function. They have been proposed to be deposits of nuclear factors and have been implicated in various cellular functions including transcriptional regulation (Doucas, 2000; Li & Chen, 2000), growth suppression (Gottifredi & Prives, 2001), and apoptosis (Quignon et al., 1998; Torii et al., 1999; Wang et al., 1998). The PODs organisation may change in neoplastic and inflammatory diseases or under other cellular stresses, and POD-associated proteins are known to be upregulated by interferons (Maul et al., 2000). The more recent interest in PODs, however, is due to their alteration in pathological situations such as following virus infection. Specific proteins of many DNA viruses are targeted to PODs, inducing their destruction and/or complete reorganisation. These include the IE1 protein of cytomegalovirus (Korioth et al., 1996; Ahn et al., 1998), ICP0 of herpes simplex

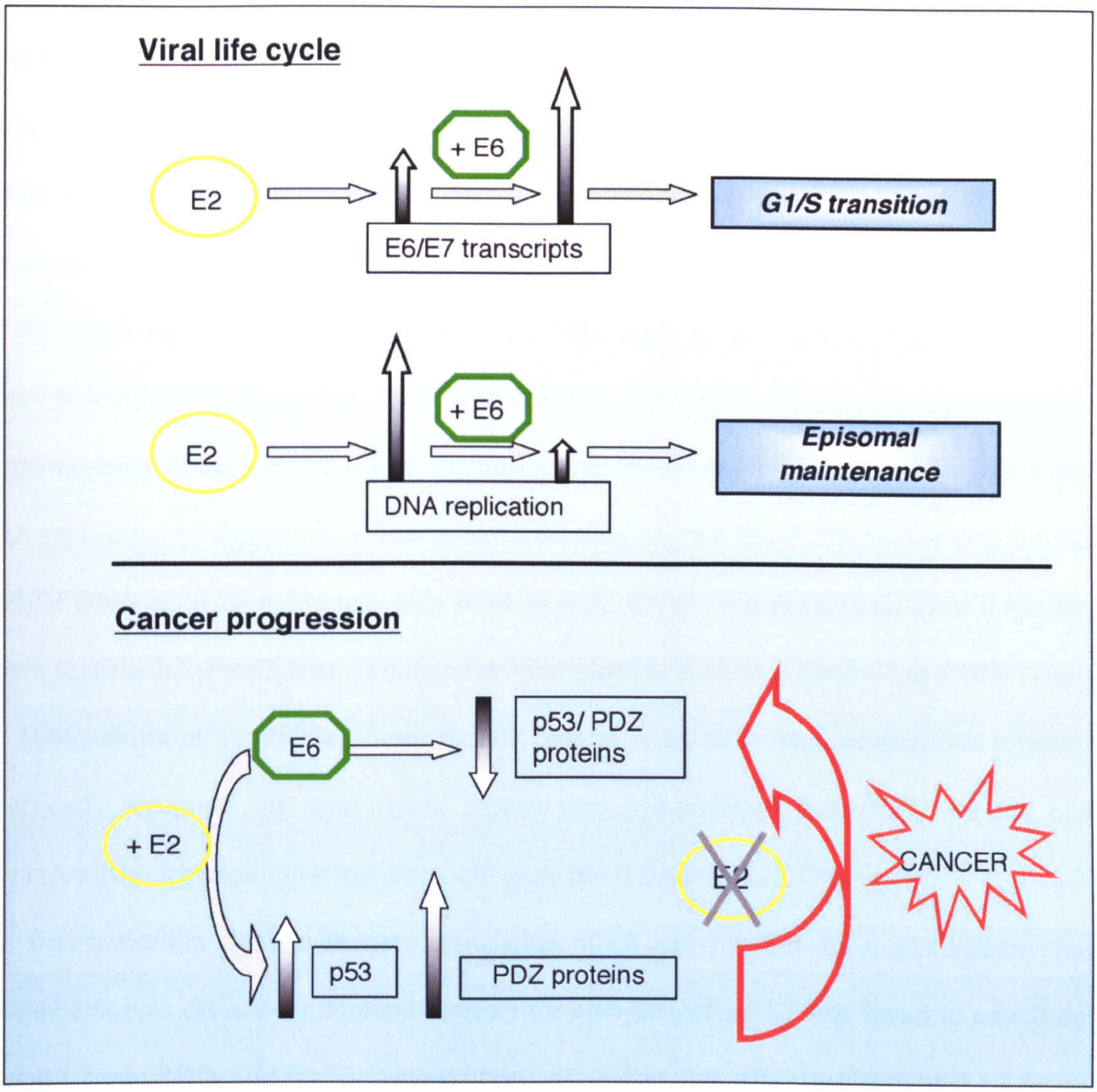


Figure 42. Schematic diagram showing potential pathways of interaction between E2 and E6.

The two major functions of E2, viral gene expression and DNA replication are modified by E6. In the normal viral life cycle the presence of E6 inhibits viral DNA replication, whilst the transcriptional activity of E2 is elevated (upper panel). This interrelationship between E2 and E6 proteins could form a regulatory loop, whereby the virus keeps viral genome amplification under control (episomal maintenance) and prepares the replication-competent cellular environment for the vegetative replication of viral DNA (G1/S transition). Conversely, E2 blocks the E6-mediated degradation of PDZ domain-containing proteins and weakly rescues p53 (lower panel). Therefore, disruption of the E2 gene as a consequence of viral DNA integration into the cellular genome would result in deregulation of E6 activities, which could then contribute to the development of malignancy (lower panel).

virus (Everett & Maul, 1994; Parkinson & Everett, 2000), and BZLF1 of Epstein-Barr virus (Adamson & Kenney, 2001), all of which interfere with the SUMO-1 modification of PML and/or Sp100, resulting in PODs disruption. The E4orf3 protein of Adenovirus associates with PML and also causes a dramatic reorganisation of PODs (Carvalho et al., 1995). Furthermore, it has also been reported that SV40 and HPV-11 DNA replicate their genomes in or near PODs (Ishov & Maul, 1996; Tang et al., 2000; Swindle et al., 1999). All of the above viral proteins are expressed early in the viral life cycle, suggesting that PODs may play a role in the initiation of DNA replication of these viruses. However, recent data suggest that PODs are not only the sites of HPV DNA replication, but may also serve as sites of virus morphogenesis since it has been shown that the L2 protein also recruits other viral proteins such as L1 and E2 to PODs (Day et al., 1998; Florin et al., 2002a). Since the E2 protein is the only viral protein that is able to specifically recognise the viral DNA, it was thus hypothesized that PODs are the main structures in which papillomavirus assembly takes place (Day et al., 1998).

The first indication that the *in vitro* association of E6 and E7 with L2 might actually have relevance *in vivo* came from immunofluorescence analysis, where L2 was found to recruit both E6 and E7 into PODs. An intriguing hypothesis arose from this data suggesting that L2 recruits the E6 and/or E7 into PODs to restrict their functions to a specific and well defined subnuclear compartment. Our idea becomes even more exciting if we take into account recent data which showed that HPV-33 L2 accumulation in PODs resulted in the reorganisation of these domains (Florin et al, 2002b; Becker et al., 2003), including recruitment of the transcriptional repressor Daxx and dispersion of the transcriptional repressor Sp100. Interestingly, the E4orf3 protein, previously shown to be responsible for redistribution of PML into tracks, also associates with and relocalises the E1B 55K protein into PODs (Leppard & Everett; Carvalho et al., 1995).

In order to gain insight into the consequences of the L2-mediated relocalisation of E6 into PODs, we first needed to investigate the effects of HPV-16 L2 upon POD organisation, since all previous studies have been carried out with HPV-33 and BPV-1 proteins (Day et al., 1998; Florin et al., 2002b). Several proteins have been found to be associated with PODs. These

include PML, the main structural component of PODs (Ishov et al., 1999; Zhong et al., 2000a); Sp100, which has been shown to influence transcription and chromatin dynamics (Seeler et al., 1998, 2001); the ubiquitin-related protein SUMO-1, which can modify PML and Sp100 (Seeler et al., 2001; Zhong et al., 2000a), and the Fas-modulating protein Daxx, which has also been shown to affect gene expression (Li et al., 2000a, b; Torii et al., 1999). In the first series of experiments we found that the HPV-16 L2 causes the redistribution of PML protein from a distinct intranuclear punctuate pattern to its accumulation in gross L2-staining nuclear bodies. Surprisingly, previous studies using BPV-1 L2 and HPV-33 L2 had shown that L2 deposits around the PML cores and does not disturb its cellular distribution (Day et al., 1998; Florin et al., 2002b). This data may indicate a functional difference between the L2 protein of HPV-16 and that of the BPV-1 and HPV-33 papillomaviruses, although it may also be due to differences in the cell types and experimental conditions used.

Increasing evidence points to alternatively spliced PML isoforms having different roles in diverse biological processes (Jensen et al., 2001; Bischof et al., 2002). Indeed, recent studies have provided evidence for different PODs with dynamic compositions within the cell, suggesting, possibly, different functions (Muratani et al., 2002; Wiesmeijer et al., 2002). In agreement with this concept, we found that the HPV-16 L2 protein is targeted to defined POD structures within the nucleus, which consist specifically of PML isoforms I, II and IV, but not PML isoforms III, V and VI. These results confirm that POD structures have different PML compositions and suggest that the viral L2 protein targets only those domains that are important for specific viral activities. This is further confirmed by the demonstration that L2 fails to colocalise with every POD within the nucleus when endogenous PML was assayed. An important question now is whether E6 affects the interplay between L2 and the different PML isoforms, and future work will address it.

In addition to PML, SUMO-1, which usually only partially colocalises with PODs, was heavily concentrated at PODs in the presence of L2. Unlike ubiquitination, in which the primary function is to target proteins for degradation, the effects of sumoylation are more substrate-

specific and can result in alteration in the function (Gostissa et al., 1999; Muller et al., 2000; Rodriguez et al., 1999), stability (Desterro et al., 1998), or intracellular location of the modified protein (Matunis et al., 1996; Zhong et al., 2000c). In addition, modification of the PML protein by SUMO is involved in the formation of PODs (Seeler & Dejean, 2001). At present the biological significance of the L2-induced redistribution of SUMO-1 into PODs is unclear, although it has the potential to have diverse effects. Since L2 contains a perfect consensus motif for sumoylation (Verger et al., 2003) it may be a substrate for SUMO-1. Moreover L2 can relocalise other viral proteins, such as L1, E2 (Day et al., 1998), E6 and E7 (this thesis) into PODs, and since some of the proteins that accumulate at PODs are or can be modified by SUMO-1 (for example PML, Sp100, Daxx), a simple mechanism would be for the viral proteins to be SUMO-1 modified at these nuclear bodies. This hypothesis is further supported by the observation that both SUMO-1 and HPV-18 E6 can also colocalise in PODs when L2 is coexpressed. Whether this represents a means by which L2 interacts with E6, or whether there are some other roles for the L2-E6 interaction in the viral life cycle remains to be determined and studies are in process to elucidate this issue.

Previous studies have shown that HPV-33 L2 could induce accumulation of Daxx within PODs (Florin et al., 2002a), and the data presented here suggest that HPV-16 L2 has a similar function. A particularly interesting aspect to these studies was the observation that L2-induced Daxx accumulation in PODs was absent in the presence of E6. Two different hypotheses can be formulated to explain these results. The simplest explanation would be that E6 causes dispersion of Daxx by interacting with L2 in PODs. An alternative explanation is that E6 induces a proteolytic degradation of Daxx when the two proteins are localised close together in the L2-staining PODs. Based on the fact that E6 targets many cellular proteins for degradation (Mantovani & Banks, 2001), the latter hypothesis seemed more likely. To address this question, the effects of proteasome inhibition were assessed in U2OS cells coexpressing L2 and E6, and this resulted in increased steady state levels of Daxx protein in the L2/E6-staining PODs. It should be noted at this point, that the distribution of Daxx in cells expressing E6 alone was

unaffected. These results suggest that L2 accumulates Daxx and E6 into PODs, a consequence of which is the E6-mediated degradation of Daxx protein in these nuclear bodies.

Whilst many pieces are still missing from the puzzle, it is nonetheless tempting to speculate on the role of E6 in degradation of L2-recruited Daxx. Daxx is a nuclear protein with multiple functions in apoptosis and transcriptional control (Michaelson, 2000). It has been identified as a repressor of basal and activated transcription (Hollenbach et al., 1999; Li et al., 2000a,b), and repression of several genes through direct interaction between Daxx and activators such Ets-1 and Pax3 has been reported (Hollenbach et al., 1999; Li et al., 2000b). The mechanism of Daxx-mediated repression has been suggested to involve recruitment of histone deacetylases to chromatin through interaction between Daxx and these enzymes (Li et al., 2000a). The L2-mediated accumulation of Daxx in PODs, putative sites of DNA virus transcription and replication (Maul, 1998), may therefore play a role in the repression of viral DNA replication and transcription in the final stage of papillomavirus assembly when the packaging of the viral minichromosome starts. On the other hand, many reports have implicated Daxx in apoptosis and its recruitment to PML nuclear bodies is suggested to be essential for its pro-apoptotic effect (Torii et al., 1999; Zhong et al., 2000b; Takahashi et al., 2004). Thus, the E6-mediated degradation of Daxx in L2-staining PODs would benefit the viral life cycle in two ways. First it would prevent the premature inhibition of the viral DNA transcription in the lower layers of the differentiated epithelium, and second it would prevent the infected cell from undergoing apoptosis at the same stage. It is also worth mentioning that disruption of PODs caused by the PML-retinoic acid receptor α fusion protein is associated with the inhibition of terminal differentiation of promyelocytes (Fagioli et al., 1994; Grignani et al., 1996). This finding raises the possibility that association of the HPV gene products with these structures influences the differentiation program of normal keratinocytes. Although induction of epithelial differentiation is normally required for capsid gene expression, it may be advantageous for the virus to delay or prevent the final stages of terminal differentiation. Therefore, examination of keratinocyte-specific differentiation markers in normal keratinocytes induced to differentiate after the

induction of L2 (or L2 plus the E6/E7 proteins that depend on L2 for POD localisation) might reveal interesting relationships among PODs, papillomavirus proteins, and epithelial differentiation. Taken together, these observations support the notion that the recruitment of viral proteins to PODs may represent a switch in the virus life cycle from a non-productive to a virus-producing, productive phase. The POD-binding proteins HSV-1 ICPO (Maul et al., 1996) and Epstein-Barr virus EBNA-5 (Szekely et al., 1996), which have been implicated in the escape from latency, have been speculated to serve an analogous function for their respective viruses, since when the reactivation lytic cycle is induced, the EBV and HHV-8 replication compartments develop in association with PML body remnants in a similar manner to that observed with HSV-1 and HCMV (Everett, 2001).

In conclusion, whilst the roles of E6/E2 and E6/L2 associations in the viral life cycle are still far from being elucidated, it is interesting to note that in the case of Adenovirus a similar complex series of viral protein interactions exists. This is best exemplified by the E1B 55K protein associations with E4orf3 and E4orf6 (Leppard & Everett, 1999). Thus the E4orf6/E1B 55K complex is required for the modulation of mRNA metabolism, and our data suggest a similar possible function for E6/E2. The role of the E4orf3 protein on the other hand is to cause the disruption of PODs and, moreover associates with and relocalise the E1B 55K into these nuclear compartments, and this has striking similarity with our observations on the L2 and E6 association. Taken together these studies suggest that different viruses have similar constrains for completing their productive infection and thereby use similar mechanisms to control their viral life cycle.

Some of the work described above is contained in the following article:

Sterlinko Grm H, Massimi P, Gammoh N & Banks L. Cross-talk between the Human Papillomavirus E2 transcriptional activator and the E6 oncoprotein. *Oncogene* 2005; in press.

PART 2: *Dissection of E6 function using small peptide inhibitors*

The E6 proteins of high-risk HPV types are known to bind cellular proteins containing similar amino acid motifs in their E6 binding domains (Chen et al., 1998; Elston et al., 1998; Vande Pol et al., 1998). E6-AP, which is the best characterized of these, functions as a ubiquitin ligase in the E6-mediated degradation of p53 (Huibregtse et al., 1993a, b). The E6 binding domain on E6-AP has been localised to an internal segment comprising amino acid residues 391-408, and an 18-mer synthetic peptide corresponding to this region has been reported to block the E6/E6-AP interaction (Huibregtse et al., 1993b). The E6-binding motif is an α-helix, and the three conserved hydrophobic residues (leucines in the case of E6-AP) used for contacting E6, form a hydrophobic patch on the same surface of the helix (Be et al., 2001). Using a random 16-mer library, two different peptides containing the L/FXELLG motif, with high homology to the E6-binding domain within E6-AP, were previously identified (Elston et al., 1998). Both peptides efficiently inhibited the interaction between E6 and E6-AP, suggesting that the E6 protein can interact with a number of related sequences through a structurally similar binding domain. To identify the precise requirements for association, and to identify reagents that might antagonise specific E6 functions, the most active peptide isolated from the original random 16-mer library, P-1 (ERWWEGVFYELLGLTE), was used as a template for the generation of a second library from which 76 binding peptides were recovered (Sterlinko Grm et al., 2004). The peptides obtained from this two-stage screen had a number of changes when compared with previously reported E6-binding peptides (Elston et al., 1998) and with the parental peptide (P-1) on which the E6-specific library was based. The hydrophobic face of the amphipathic α-helix was perfectly preserved although certain hydrophobic residues were clearly favoured at particular positions, presumably because of their size or other characteristics. Similarly, the overall charge on the hydrophilic face was found to increase in the best-binding peptides, with acidic residues generally replacing polar residues. In short, the characteristics of the amphipathic alpha helix and, hence, the binding to E6, were perfected. Selection for particular amino acids was also

apparent outside the putative helical region and, in the most-improved peptide V-1 (EGWWEGVFDELLGMAG), this may increase the length of the amphipathic helix.

Whilst these peptides were developed in John Doorbar's laboratory, we were interested in investigating their effects upon the ability of E6 to bind and to target its different substrates for proteolytic degradation. This is particularly important from a therapeutic point of view, since the development of new treatment methods based on direct antiviral therapy would clearly be a useful addition to the currently available approaches in clinical management. To perform this study we used the most active peptide, P-1, identified in the first study (Elston et al., 1998) and the most improved peptide, V-1, obtained from the second screen (Sterlinko Grm et al., 2004). The sequences of both peptides, plus the sequence of the jumbled peptide used as a negative control (cont-1), are shown in Figure 43. The sequence of the E6-AP peptide (P-E6-AP), which corresponds to the E6-binding domain within E6-AP is also shown for comparison.

Peptides P-1 and V-1 reduce the ability of HPV-16 E6 and HPV-18 E6 to target p53 for degradation

The most famous and most studied target of high-risk HPV E6 is the tumour suppressor, p53 (Thomas et al., 1999b for review). In this interaction, E6 binds to p53 together with a cellular protein, E6-AP (Huibregtse et al., 1991, 1993a; Scheffner et al., 1993), which functions as a ubiquitin ligase, and p53 is subsequently polyubiquitinated and degraded at the 26S proteasome (Scheffner et al., 1990; Werness et al., 1990). We wanted to test first whether the two synthetic peptides P-1 and V-1, described above, could affect the interaction between E6, E6-AP and p53. To do this, GST pulldown assays were performed using purified GST-E6-AP, GST-p53 and GST alone in the presence of either P-1 or V-1 and *in vitro* translated radiolabelled HPV-16 E6. The E6 was translated in both rabbit reticulocyte lysate and wheat germ extract, to exclude any effects of endogenous E6-AP, which is present in rabbit reticulocyte lysate but not in wheat germ extracts. The results obtained are shown in Figures 44 and 45. As can be seen, in the rabbit reticulocyte lysate system, V-1 and P-1 effectively block the interaction between E6 and E6-AP

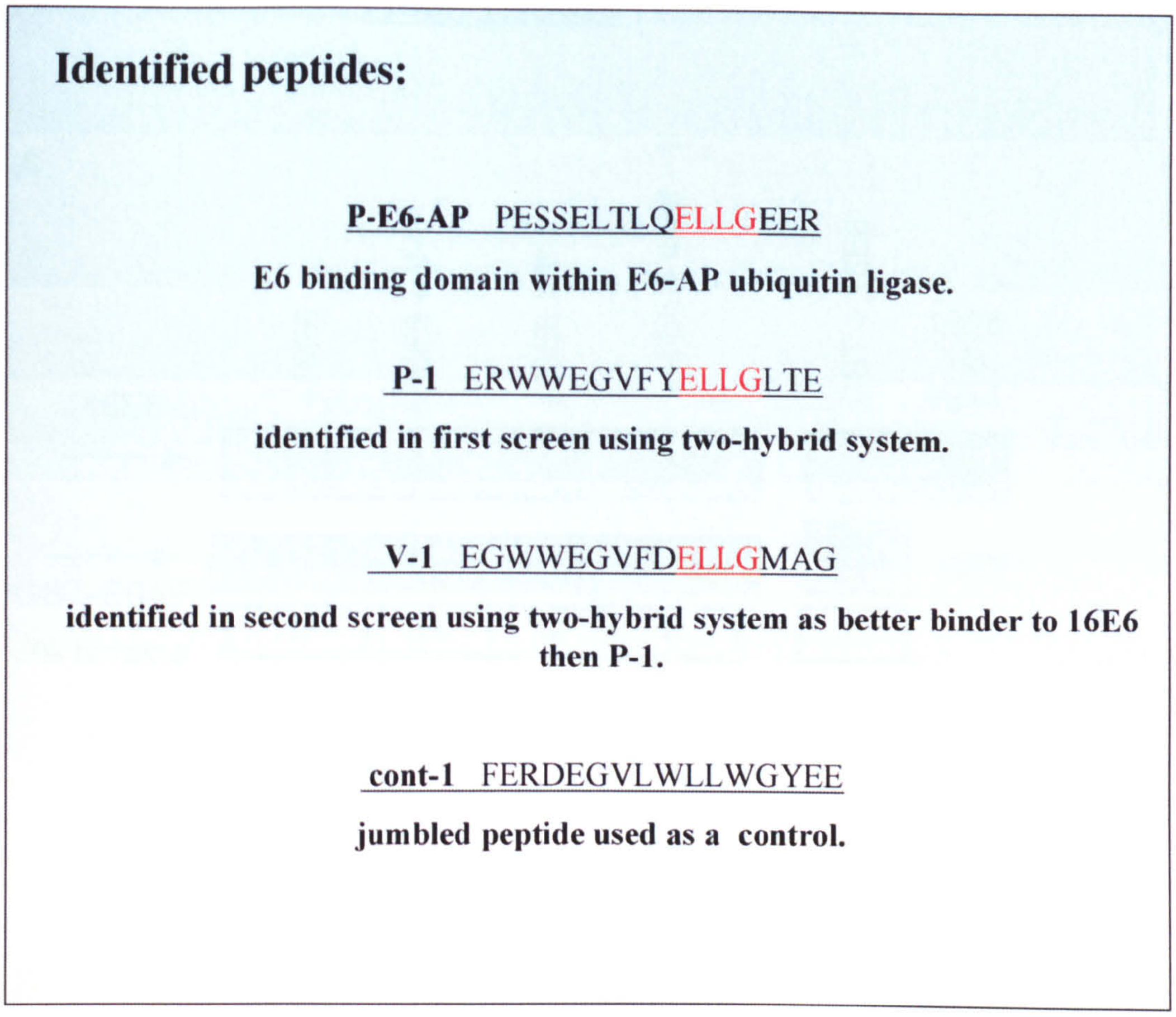


Figure 43. Amino acid sequences of the E6 binding synthetic peptides used in the study.

Peptide P-E6-AP peptide corresponds to the E6 binding domain within the E6-AP ubiquitin ligase. The P-1 was isolated from a random 16-mer peptide library in a yeast two-hybrid system as an HPV-16 E6 binding peptide. This peptide was further used as a template for the generation of a second library from which the peptide V-1, defined as a better binder to E6 than the parental peptide (P-1), was recovered. The scrambled peptide was used as a negative control (cont-1). The ELLG motif present within peptides P-E6-AP, P-1, and V-1 is shown in red.

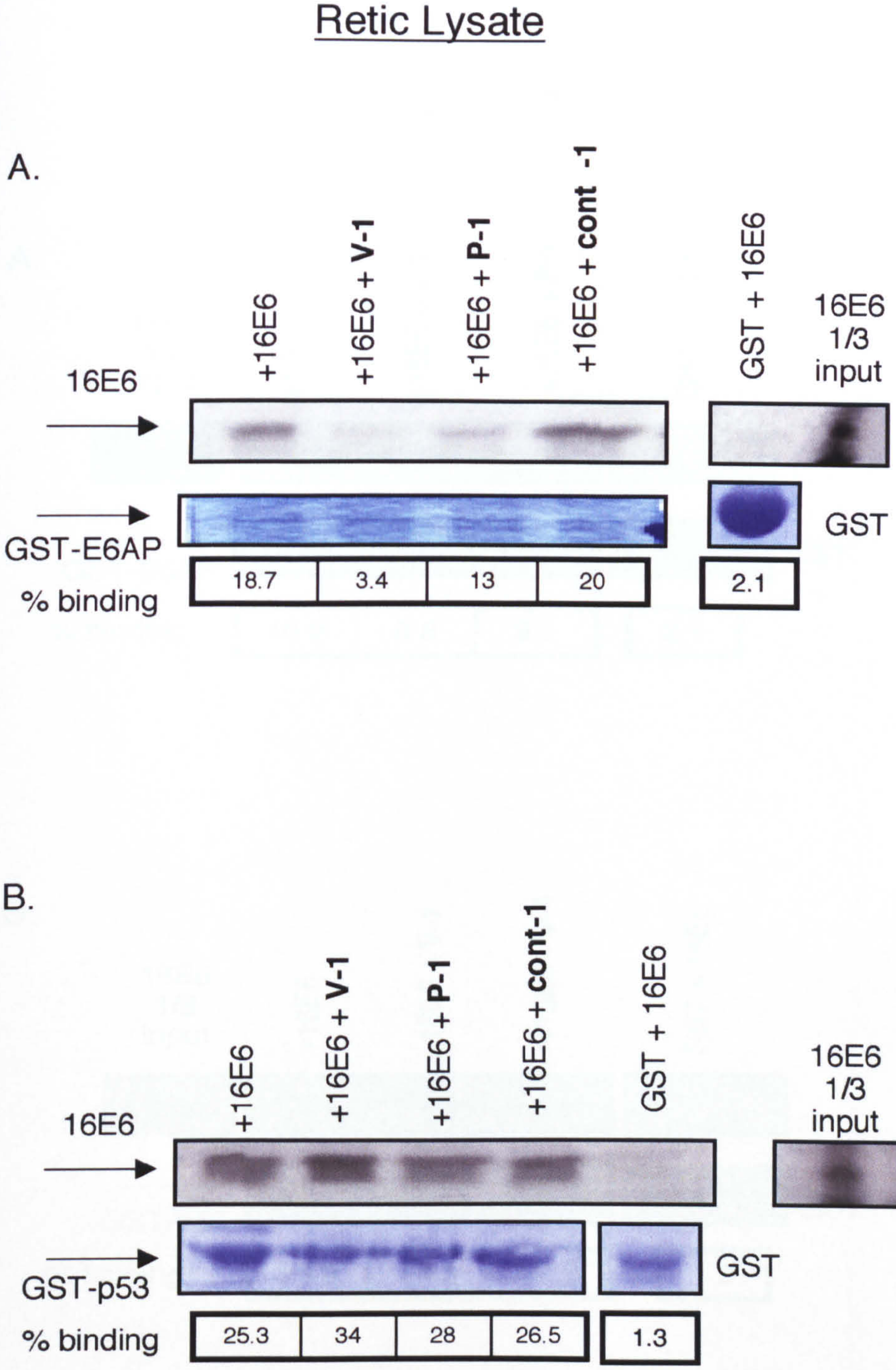


Figure 44. The effect of peptides V-1 and P-1 (1mM) on the binding of HPV-16 E6 to GST-E6AP and to GST-p53 in reticulocyte lysate.
(A) Radiolabelled *in vitro* translated E6 and the peptide, as indicated, were pre-incubated for 20 minutes at room temperature and then incubated with GST-E6-AP or GST alone as a control. Following extensive washing, bound E6 proteins were visualised using SDS-PAGE and autoradiography. (B) Radiolabelled *in vitro* translated E6 and the peptide, as indicated, were pre-incubated for 20 minutes at room temperature and then incubated with GST-p53 or GST alone as a control. Following extensive washing, bound E6 proteins were visualised using SDS-PAGE and autoradiography. The bottom panels show the Coomassie stain of the GST fusion proteins. Protein inputs for the assay are also shown (right panels). Numbers below each panel show the percentage of bound protein. Representative results of three experiments are shown.

Figure 45. The effect of the peptides V-1 and P-1 (1mM) on the binding of HPV-16 E6 to GST-E6AP and to GST-p53 in the absence of endogenous E6-AP (wheat germ).
(A) Radiolabelled *in vitro* translated E6 and the peptide, as indicated, were pre-incubated for 20 minutes at room temperature and then incubated with GST-E6-AP or GST alone as a control. Bound E6 proteins were visualised using SDS-PAGE and autoradiography. (B) Radiolabelled *in vitro* translated E6 and the peptide, as indicated, were pre-incubated for 20 minutes at room temperature and then incubated with GST-p53 or GST alone as a control. Following extensive washing, bound E6 proteins were visualised using SDS-PAGE and autoradiography. After exposure, the gels were rehydrated and stained with Coomassie brilliant blue to show GST-fusion protein inputs (lower part of each panel). Numbers below each panel show the percentage of bound protein. Representative results of at least three experiments are shown.

whilst the scrambled control peptide cont-1 has no effect (Figure 44A). The same result was obtained using the wheat germ expression system (Figure 45A). In contrast, neither peptide has any effect upon the interaction between E6 and p53 (Figures 44B and 45B), suggesting that E6-AP binding is not a prerequisite for the interaction with p53, and this is in agreement with previous studies (Crook et al., 1991a; Lechner & Laimins, 1994; Li and Coffino, 1996).

Having shown that V-1 and P-1 can abolish the interaction between E6 and E6-AP, but not that between E6 and p53, we were then interested in investigating the effects of these peptides upon the ability of E6 to target a number of its substrate proteins for proteasome-mediated degradation. To do this we first assessed the effects of the two peptides upon p53 degradation. *In vitro* translated HPV-16 E6 was first pre-incubated with the peptides, and then added to *in vitro* translated p53. After 30 min at 30°C the residual p53 protein was determined by immunoprecipitation. The results obtained are shown in Figure 46A. As can be seen, p53 is effectively degraded by E6 over the course of the assay. However, both V-1 and P-1 abolish the ability of E6 to degrade p53, consistent with their being able to block recruitment of E6-AP. No inhibition is observed with the control peptide (cont-1).

Previous studies have shown that HPV-16 E6 binds somewhat more strongly to p53 than HPV-18 E6, and concomitantly degrades p53 more efficiently (Scheffner et al., 1990). We were therefore interested in determining whether there was any difference in how the peptides inhibited HPV-18 or HPV-16 E6-mediated degradation of p53. To examine this, we repeated the degradation assay, using *in vitro* translated HPV-18 E6. The results obtained are shown in Figure 46B and demonstrate that there is no difference between the two oncoproteins. Taken together, these results demonstrate that both V-1 and P-1 are able to inhibit the E6-directed degradation of p53, with no difference between HPV-18 and HPV-16 E6 (Figure 46, compare A with B).

We then proceeded to investigate whether there were any differences in the efficiency with which the peptides could block E6-induced degradation of p53. To investigate this, we performed an *in vitro* degradation assay, as described previously, using different concentrations

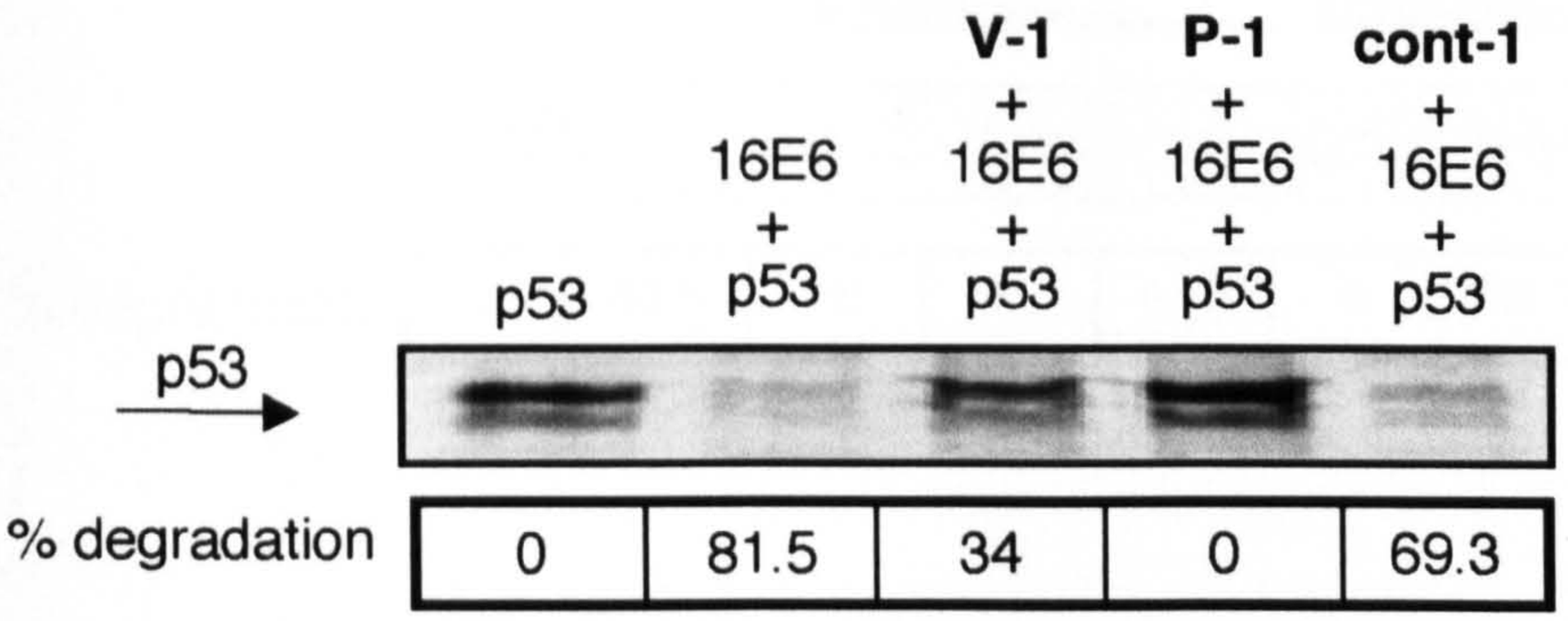
of peptides (1, 0.5 and 0.1 mM). The mixture was then incubated at 30°C for 30 min and the results are shown in Figure 47. The parental peptide P-1 is the most effective in this assay since it completely blocks HPV-16 E6-induced degradation of p53 at 1mM, whilst V-1 at the same concentration still allowed 60% of the p53 to be degraded. Broadly similar results were obtained with HPV-18 E6 (Figure 47, lower panel). These results demonstrate that P-1 is a more effective inhibitor of E6-induced p53-degradation than V-1.

Inhibition of HPV-18 E6-mediated degradation of Dlg

Having determined that the two α-helical-binding peptides could effectively block E6-AP binding as well as E6-induced p53 degradation, we went on to assess their abilities to inhibit E6-mediated degradation of its PDZ domain-containing substrates. Unlike the E6/E6-AP interactions with its substrates, the PDZ domain-containing substrates are targeted by the high-risk E6 proteins using a four amino acid binding motif at the extreme C-terminus (Lee et al., 1997; Gardiol et al., 1999; Pim et al., 2000). In addition, although some of these targets appear to be degraded in an E6-AP-dependent fashion, there are others which appear to be targeted independently of E6-AP (Pim et al., 2000).

We first investigated the effects of the peptides upon E6-mediated degradation of Dlg. The assays were performed as for p53, except that the degradation assay requires two hours at 30°C. The results obtained are shown in Figure 48A. As can be seen, the E6-mediated degradation of Dlg is inhibited by the two peptides in a manner similar to that of p53 since both V-1 and P-1 inhibit HPV-18 E6-mediated degradation of the Dlg protein. To verify that the peptides were not affecting the interaction between HPV-18 E6 and Dlg, we performed an *in vitro* binding assay with GST-Dlg and *in vitro* translated HPV-18 E6 in the presence of the peptides. As can be seen from Figure 48B, the binding of Dlg to HPV-18 E6 was not disrupted by either peptide. When taken together, these results suggest that the peptides bind a region of E6 that is required for E6-induced degradation of both p53 and Dlg.

A.



B.

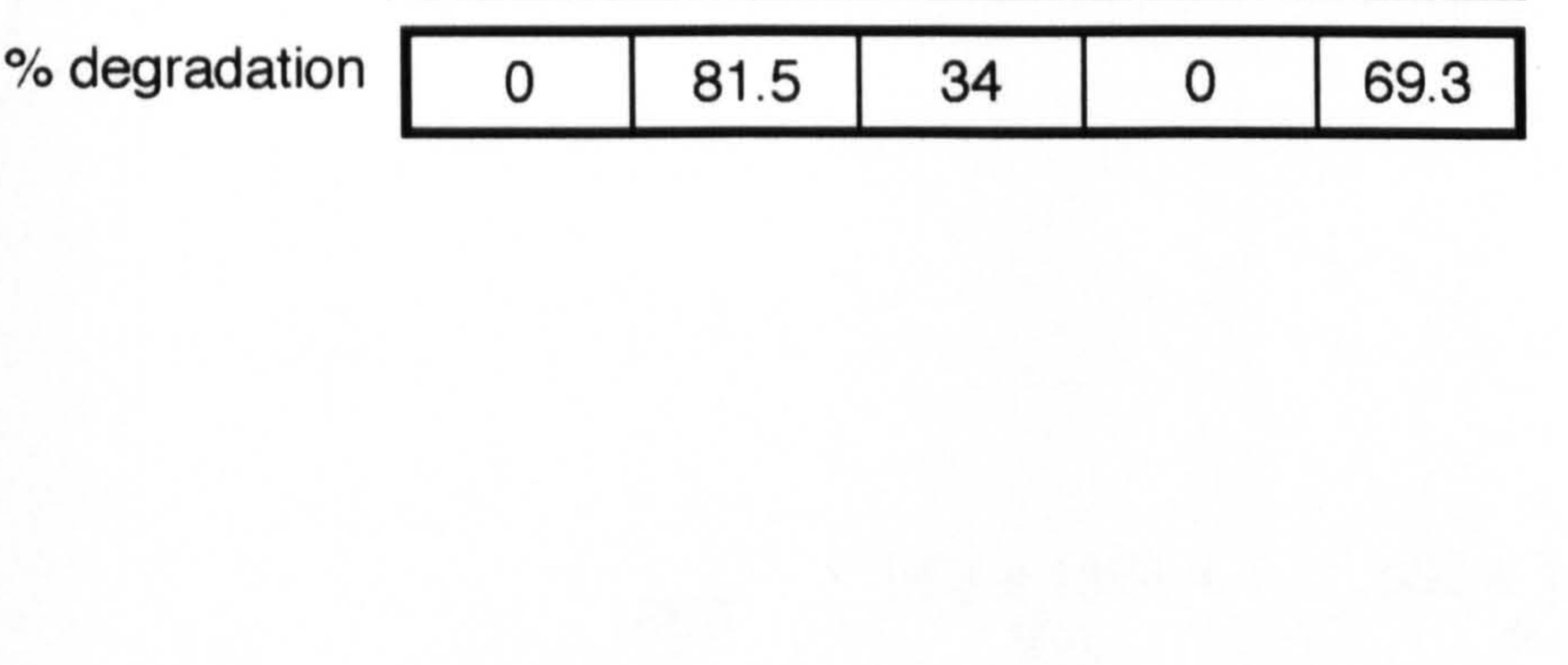


Figure 46. Peptides V-1 and P-1 can specifically inhibit HPV-16 (A) and HPV-18 (B) E6-directed degradation of p53 in vitro.
The *in vitro* translated HPV-16 or HPV-18 E6 proteins were pre-incubated with V-1 (1mM), P-1 (1mM) or control peptide (cont-1) (1mM) at room temperature for 20 minutes prior to the addition of *in vitro* translated p53. The remaining target protein was detected by immunoprecipitation, followed by SDS-PAGE and autoradiography. The positions of p53 is indicated. The numbers below each lane show the percentage of input p53 that is degraded. The representative result of at least three experiments is shown.

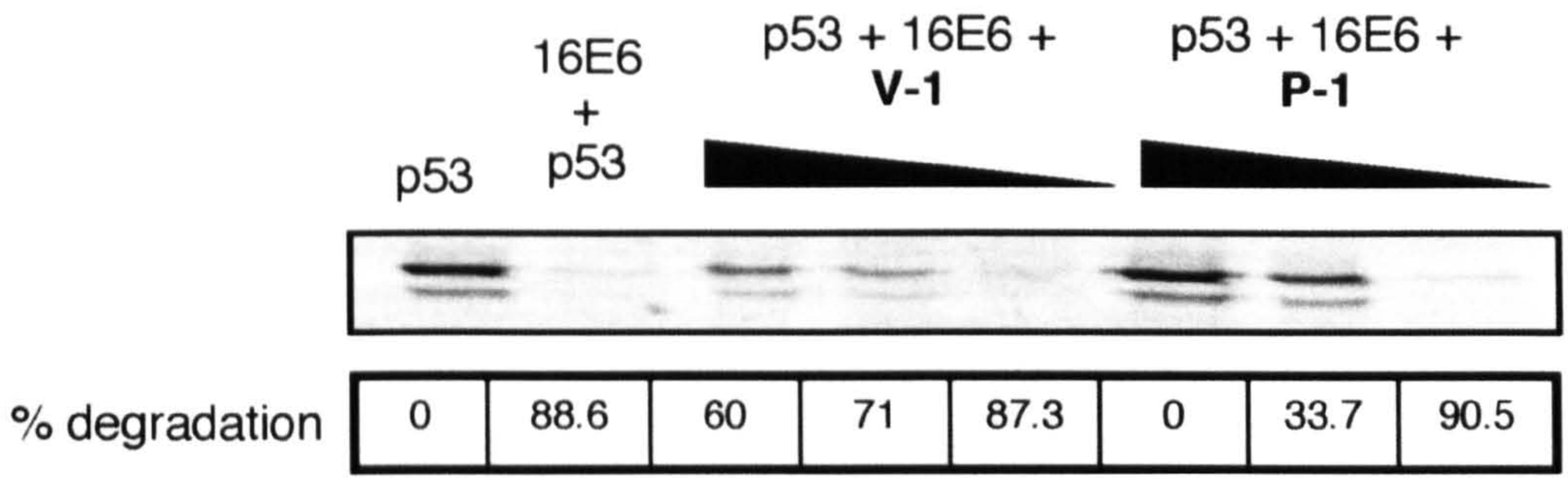
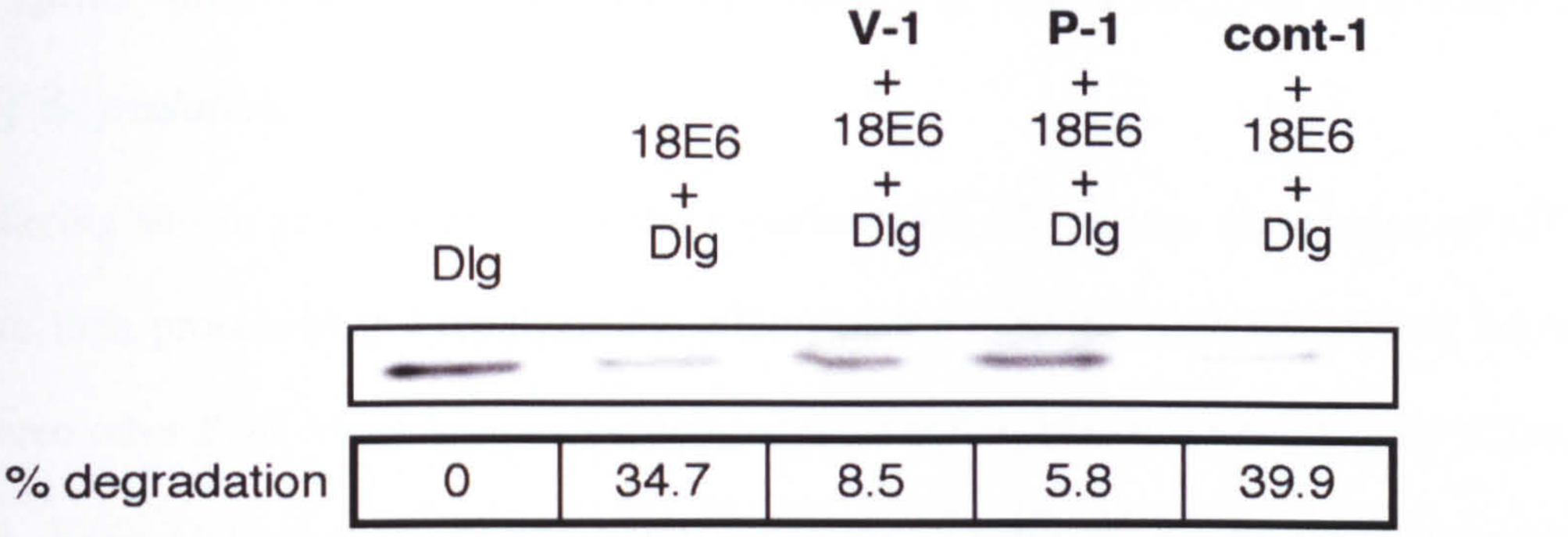


Figure 47. Peptide P-1 is more efficient than V-1 in inhibiting E6-induced degradation of p53.

The *in vitro* degradation of p53 protein with radiolabelled HPV-16 E6 (upper panel) and HPV-18 E6 (lower panel) was performed in the presence of decreasing concentrations of peptide (1, 0.5 or 0.1 mM, left to right). The amount of p53 remaining was assessed by SDS-PAGE and autoradiography. The position of p53 is indicated. The numbers below each lane show the percentage of input p53 that is degraded. The representative result of at least three experiments is shown.

A.



B.

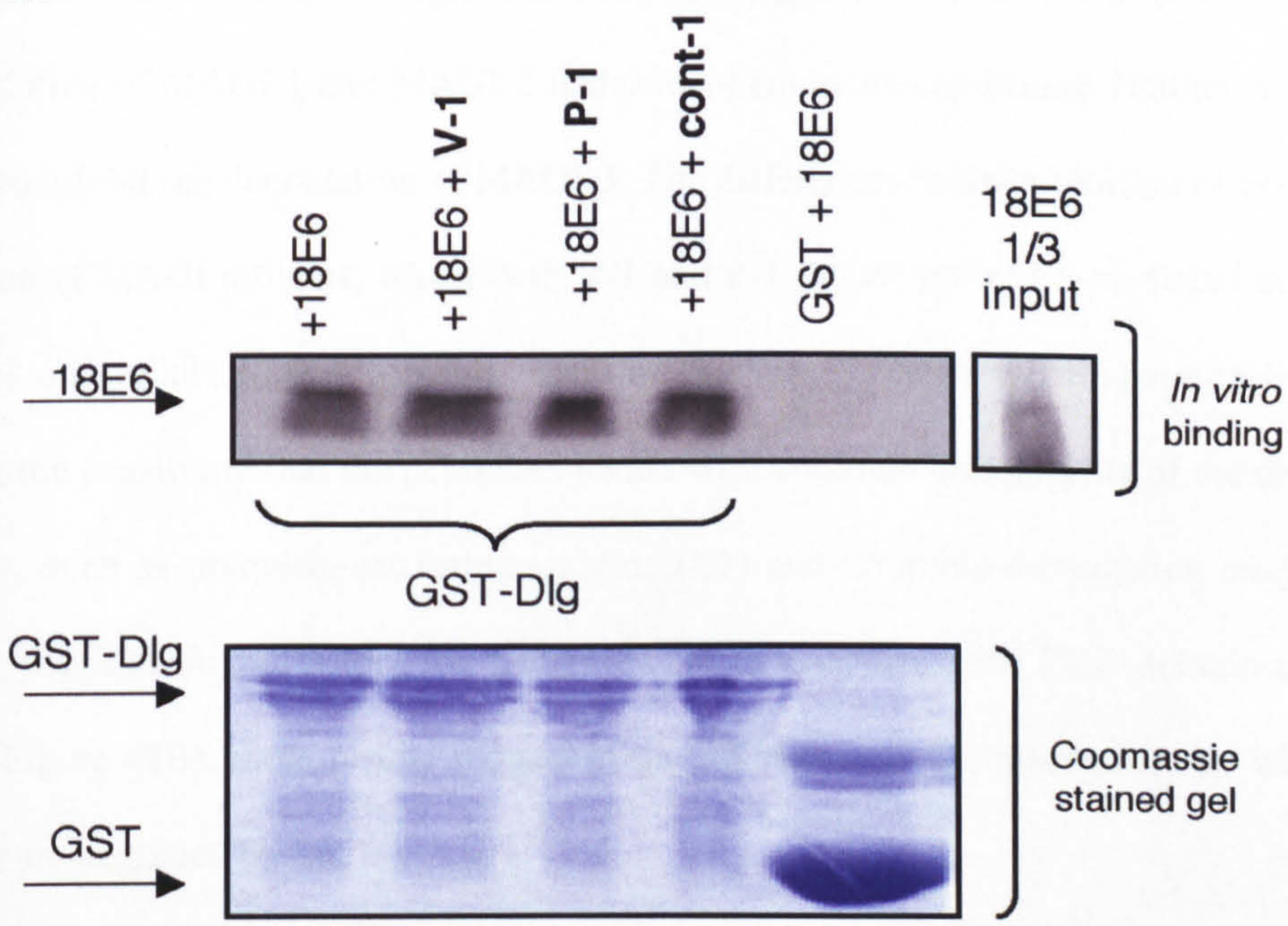


Figure 48. HPV-18 E6-mediated degradation of Dlg is inhibited by peptides V-1 and P-1.

(A) *In vitro* translated Dlg was incubated in the presence of *in vitro* translated HPV-18 E6 alone or with the peptides (1mM), as indicated, for two hours. The presence of Dlg was determined by immunoprecipitation followed by SDS-PAGE and autoradiography. The arrow indicates the position of Dlg and the numbers show the percentage of input Dlg that is degraded. (B) A binding assay between GST-Dlg and *in vitro* translated HPV-18 E6 in the presence and absence of the V-1, P-1, and control (cont-1) peptides. As can be seen the peptides have no significant effect on E6 binding to Dlg. The bottom panel shows the Coomassie stain of the GST fusion proteins. The representative results of at least three experiments are shown.

Peptide inhibition of E6-induced degradation of MAGI suggests alternative pathways of degradation

Having shown similarities in how the peptides block E6-induced degradation of p53 and Dlg, we then proceeded to investigate the effects of the peptides upon E6-induced degradation of three other PDZ domain-containing substrates, MAGI-1, MAGI-2 and MAGI-3 (Glaunsinger et al, 2000; Thomas et al., 2001b, 2002). To do this, a series of *in vitro* degradation assays were performed, using *in vitro* translated MAGIs plus HPV-18 E6, and the results obtained are shown in Figure 49. As can be seen, the P-1 peptide is capable of inhibiting E6-induced degradation of MAGI-1, but has no effect on E6-induced degradation of either MAGI-2 or MAGI-3. Peptide V-1, which is able to inhibit the degradation of p53 (Figure 46), has only a poor ability to block the degradation of MAGI-1 and MAGI-2 under identical assay conditions. Neither V-1 nor P-1 was able to inhibit the degradation of MAGI-3. The differences in the inhibition of E6-mediated degradation of MAGI proteins, where both V-1 and P-1 fail to inhibit E6-mediated degradation of at least one of the MAGI proteins, confirm the specificity of the E6/peptide interaction, excluding the possibility that the peptides interact with common components of the degradation machinery, such as ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2). Since the peptides do not affect the ability of E6 to complex with PDZ domain-containing proteins (Figure 48B), these results suggest basic differences in the mechanism by which these substrates are degraded by E6.

HPV-16 E6-induced degradation of MAGIs is susceptible to peptide P-1 inhibition

Since the P-1 peptide did not block HPV-18 E6-mediated degradation of MAGI-2 or MAGI-3, we then proceeded to investigate the effects of this peptide upon the HPV-16 E6-induced degradation of the MAGI proteins. Degradation assays were performed, as above, using *in vitro* translated MAGI proteins plus HPV-16 E6, and the results are shown in Figure 50. As expected, the degradation of MAGI-1 induced by HPV-16 E6 (Figure 50, upper panel) is inhibited by the P-1 peptide in a manner similar to that seen with HPV-18 E6 (Figure 49, upper panel).

However, whilst P-1 is incapable of blocking HPV-18 E6-induced degradation of MAGI-2 and MAGI-3 (Figure 49, middle and bottom panels) it is nonetheless a potent inhibitor of HPV-16 E6-induced degradation of these substrates (Figure 50, middle and bottom panels). These results suggest that there may also be differences in the mechanisms by which the HPV-16 and HPV-18 E6 proteins target MAGI-2 and MAGI-3 for degradation.

The prototype E6-AP peptide (P-E6-AP) does not inhibit E6-directed degradation of its cellular targets

It has been previously shown that an 18-mer or 16-mer synthetic peptide corresponding to the E6 binding domain within E6-AP would block the interaction between E6 and E6-AP (Huibregtse et al., 1993b; Elston et al., 1998). Based on these observations we wanted to evaluate the effects of this peptide (P-E6-AP) upon the ability of E6 to bind and to target its various substrates for proteolytic degradation, and compare them with the above data on the V-1 and P-1 peptides. Since this peptide is based on the prototype E6-AP interaction motif we first analysed its effect upon the E6/E6-AP and E6/p53 interactions. To do this, GST pulldown assays were carried out using purified GST-E6-AP, GST-p53 and GST alone in the presence of P-E6-AP and *in vitro* translated HPV-16 E6. As described above, this was done using E6 translated in either rabbit reticulocyte lysate or wheat germ extract. The results obtained are shown in Figure 51. As expected from our previous results, the P-E6-AP peptide does not inhibit the interaction between E6 and p53 (Figure 51B). Surprisingly, however, the binding of HPV-16 E6 to E6-AP was not disrupted by the P-E6-AP peptide, regardless of whether the assays were performed in the presence (reticulocyte lysate) or absence (wheat germ) of endogenous E6-AP (Figure 51A). In contrast, V-1 and P-1 effectively blocked E6/E6-AP interaction under identical assay conditions (Figures 44A and 45A).

While we did not observe an effect of P-E6-AP on the E6/E6-AP interaction, we considered it possible that it might have an influence on E6-mediated degradation of its cellular targets. To investigate this possibility, degradation assays were performed, as before, using *in vitro*

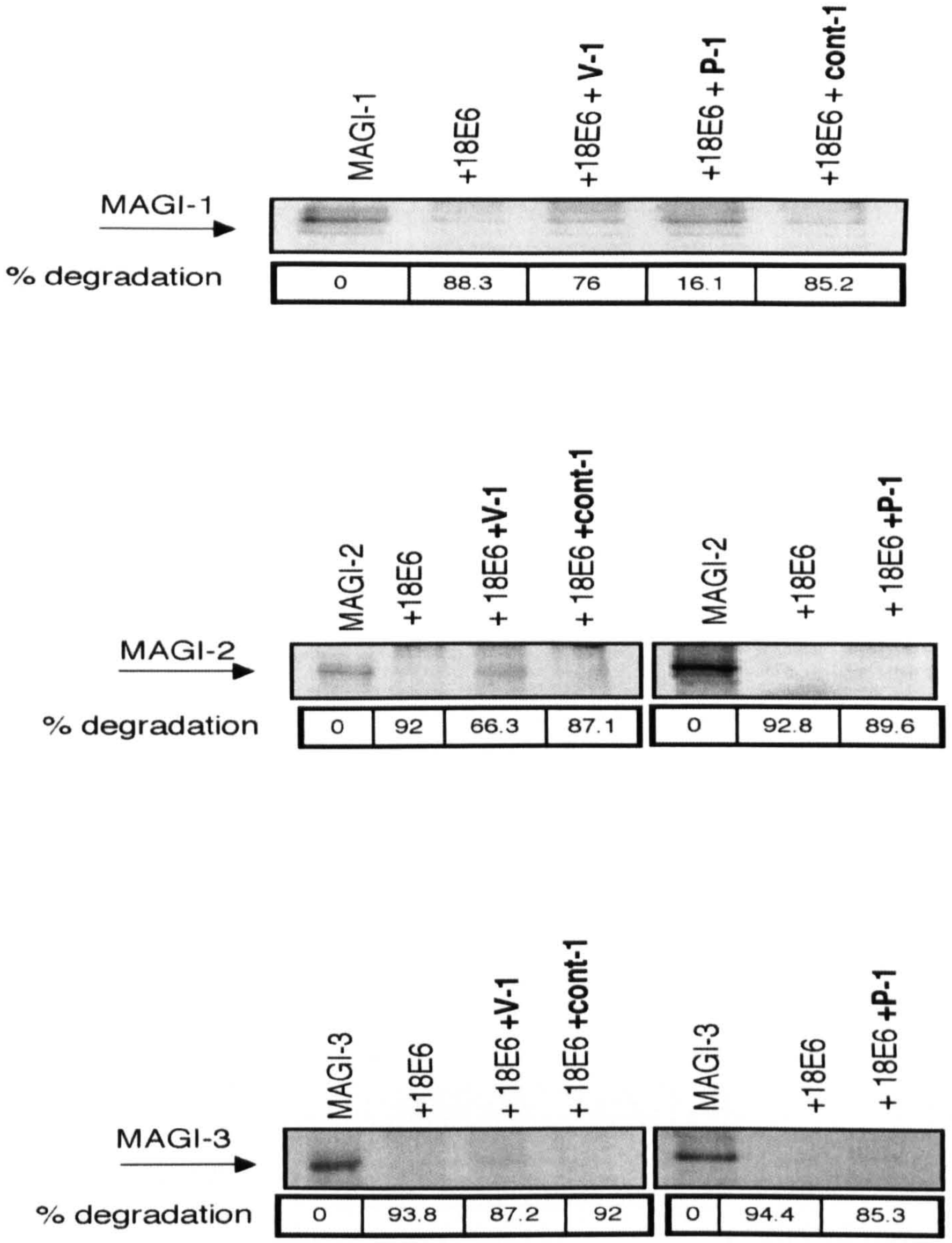


Figure 49. Peptide inhibition of HPV-18 E6 degradation of MAGI-1, MAGI-2 and MAGI-3.

The V-1, P-1 and control peptides (cont-1) at 1 mM concentration were pre-incubated with *in vitro* translated HPV-18 E6, as indicated, prior to adding *in vitro* translated MAGI-1, MAGI-2 or MAGI-3 protein. After one hour, residual protein was detected by immunoprecipitation using a polyclonal anti-WW antibody followed by SDS-PAGE and autoradiography. The numbers show the percentage of input protein that is degraded. The representative result of at least three experiments is shown.

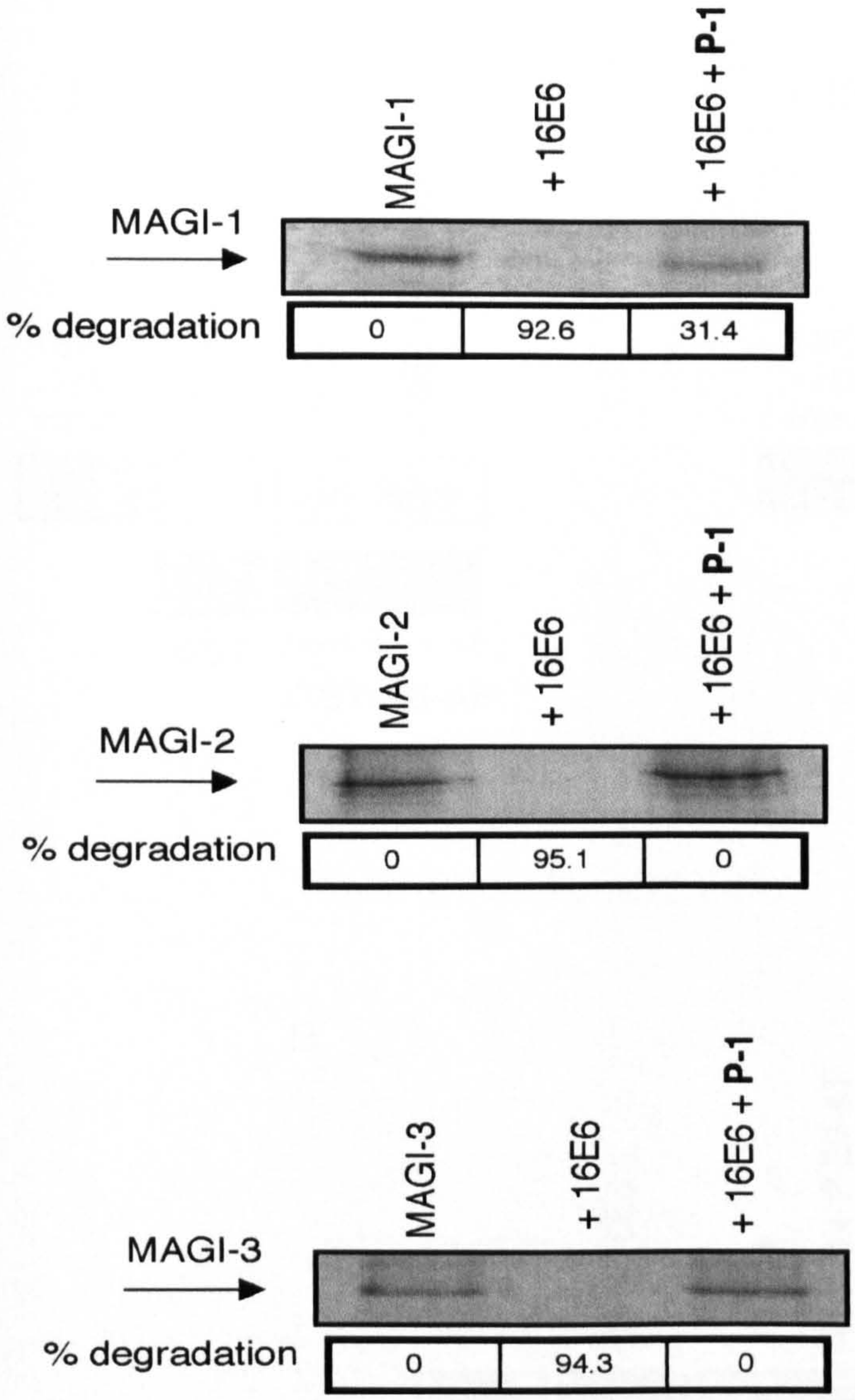


Figure 50. Inhibition of HPV-16 E6-mediated degradation of MAGI proteins by peptide P-1.

In vitro translated MAGI proteins were incubated with *in vitro* translated HPV-16 E6 proteins, pre-incubated in the presence or absence of the P-1, for 60 minutes at 30°C. Remaining protein was determined by immunoprecipitation followed by SDS-PAGE and autoradiography. The arrows indicate the positions of MAGI-1, MAGI-2, and MAGI-3 proteins and the numbers below each panel show the percentage of input protein that is degraded. The representative results of at least three experiments are shown.

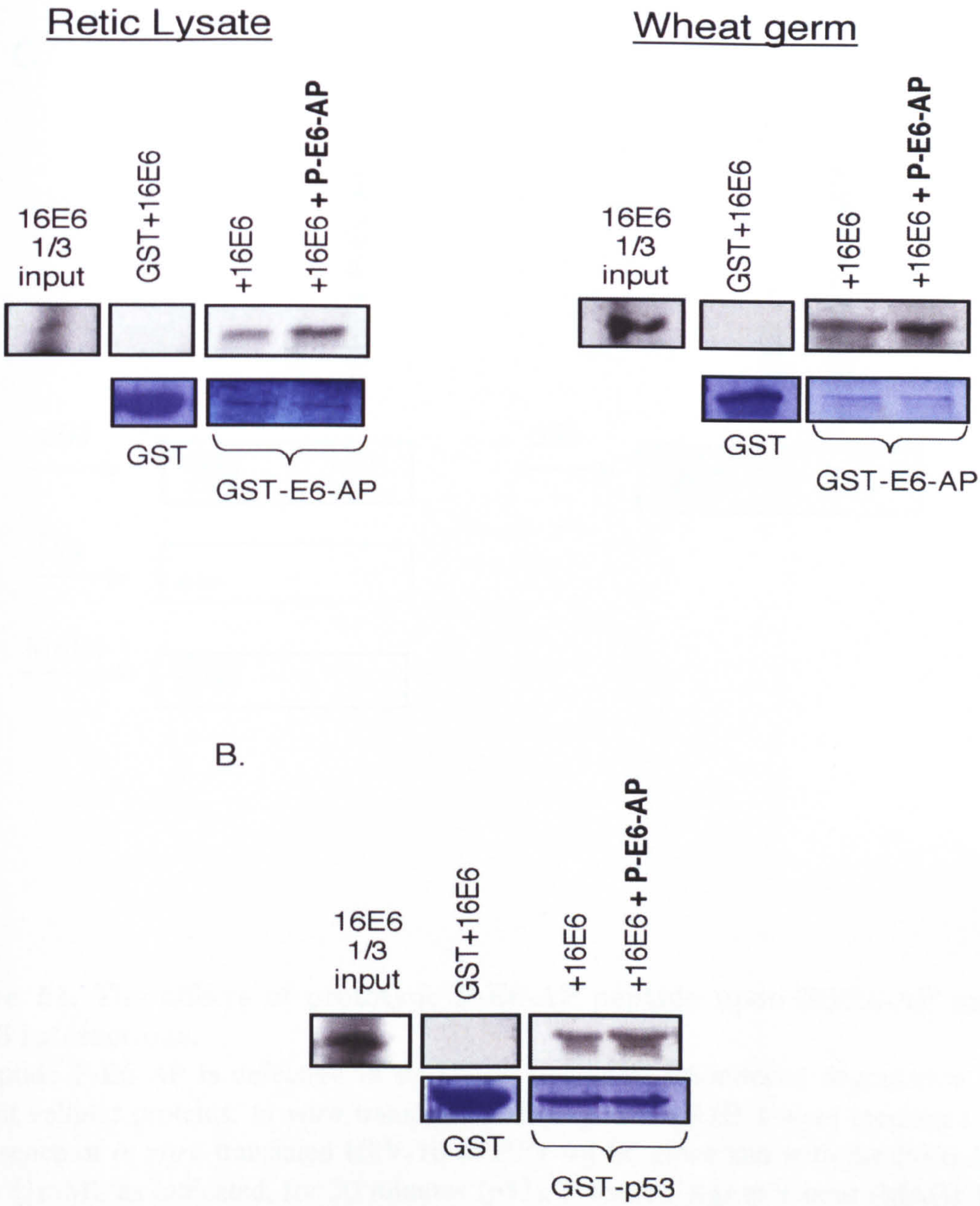


Figure 51. The effects of prototype P-E6-AP peptide upon E6/E6-AP and E6/p53 interactions.

(A) A binding assay between GST-E6-AP and *in vitro* translated HPV-16 E6 using either rabbit reticulocyte lysate or wheat germ extract, as indicated, in the presence and absence of the P-E6-AP peptide. Bound proteins were visualised using SDS-PAGE and autoradiography. After exposure, the gels were rehydrated and stained with Coomassie brilliant blue to show GST-fusion protein inputs (lower part of each panel). (B) A binding assay between GST-p53 and *in vitro* translated HPV-16 E6 using rabbit reticulocyte lysate, in the presence and absence of the P-E6-AP peptide. Bound proteins were visualised using SDS-PAGE and autoradiography. After exposure, the gels were rehydrated and stained with Coomassie brilliant blue to show GST-fusion protein inputs (lower part of each panel). The results are part of the same set of experiments shown in Figures 44 and 45.

C.

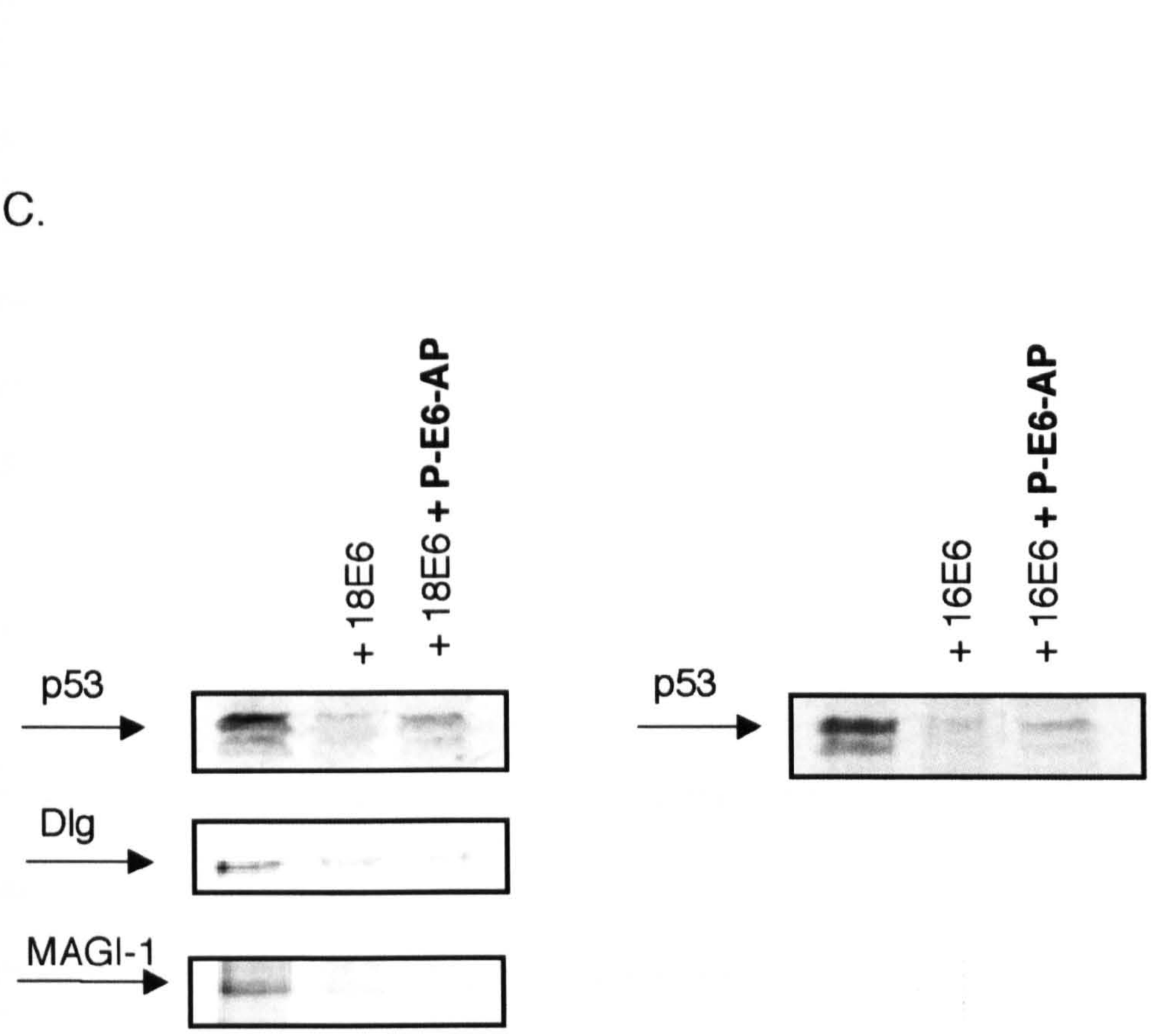


Figure 51. The effects of prototype P-E6-AP peptide upon E6/E6-AP and E6/p53 interactions.

(C) Peptide P-E6-AP is defective in its ability to inhibit E6-induced degradation of different cellular proteins. *In vitro* translated p53, Dlg and MAGI-1 were incubated in the presence of *in vitro* translated HPV-18 or HPV-16 E6 alone and with the P-E6-AP peptide (1mM), as indicated, for 30 minutes (p53), 2 hours (Dlg) or 1 hour (MAGI-1). The presence of each protein was determined by immunoprecipitation, followed by SDS-PAGE and autoradiography. The results are part of the same set of experiments shown in Figures 46A and B, 48A and 49.

translated p53, Dlg and MAGI-1 proteins in the presence of *in vitro* translated E6, which had been pre-incubated with P-E6-AP peptide. The results obtained are shown in Figure 51C and demonstrate again that, whilst V-1 and P-1 can inhibit the E6-directed degradation of all these substrates (Figures 46, 48 and 49), the P-E6-AP peptide is largely defective for this activity (Figure 51C). These results indicate that only higher affinity peptides, such as V-1 and P-1 are effective in our assays, suggesting that the basal interaction between E6 and E6-AP may be rather weak, and possibly transitory.

Peptide P-5 does not block the E6/E6-AP interaction, but still inhibits E6-mediated degradation of p53

Comparison of the V-1 and P-1 peptide sequences with P-E6-AP peptide revealed homology within the carboxy terminal portion of the three peptides, which is characterised by the presence of an ELLG motif (Figure 43). In contrast, comparison of the amino terminal halves of the peptides reveals no homology (Figure 43). Hence, one of the possible explanations for the discrepancy in the ability of V-1 or P-1 and P-E6-AP peptides to inhibit the E6/E6-AP interaction and E6-mediated degradation of its cellular targets could be that the N-terminal region of these peptides is responsible for the observed inhibition. To address this question we designed a new peptide, P-5, consisting of the N-terminal half of the most active peptide in our assays, P-1, and the C-terminal half of the control peptide (cont-1). As can be seen in Figure 52A, peptide P-5 does not contain the **E/D-L/I/F-L/V-G** motif, shown to be important for the interaction with E6, and we were therefore interested in determining whether this peptide can still bind to E6. To investigate this, an *in vitro* avidin pulldown assay was performed. The *in vitro* translated E6 proteins were first pre-incubated with biotinylated peptide P-5, and the E6 protein bound to the biotinylated P-5 was recovered by the addition of avidin-agarose resin which was gently mixed with the proteins for 20 minutes. The resin was then washed and the bound proteins were analysed by SDS-PAGE. Figure 52B shows that both HPV-16 and HPV-18 E6 were recovered when the biotinylated P-5 peptide was present in the reaction. These results

demonstrate that peptide P-5, even without the ELLG motif, is still able to bind to the E6 oncoproteins.

Having shown that P-5 interacts with the E6 proteins we were interested in examining its effect on the ability of E6 to interact with its different substrates and to target p53 for degradation. We therefore performed a series of *in vitro* binding assays, as described above, with GST-E6-AP or GST-p53 and *in vitro* translated HPV-16 E6 in the presence of the peptide P-5. As can be seen from Figure 52C, the P-5 peptide does not inhibit either the interaction between E6 and E6-AP or the interaction between E6 and p53. We next assessed the ability of P-5 to abrogate the E6-mediated degradation of p53, by performing *in vitro* degradation assays as described previously, with the difference that E6 proteins were pre-incubated with P-5. The results are shown in Figure 52D. Surprisingly, the P-5 peptide, while having no affect on the ability of E6 to bind E6-AP and p53, is nonetheless a potent inhibitor of E6-induced degradation of p53. Taken together these results suggest a far more complex pattern of interactions than the simple recruitment of the E6-AP ubiquitin ligase by E6 for the E6-directed degradation of p53.

E6-induced degradation of Dlg and MAGIs is E6-AP independent

The above results indicate that there are significant differences in how E6 targets p53, Dlg, and the MAGI proteins for degradation, and they raise the possibility that E6-AP may not be involved for all of them. To investigate this further we performed a series of *in vitro* E6-AP immunodepletion assays to determine whether there are any differences in the E6-induced degradation of these targets in the presence or absence of E6-AP. The HPV-16 and HPV-18 E6, p53, Dlg and MAGI proteins were *in vitro* translated using the rabbit reticulocyte system, and then incubated with rabbit polyclonal anti-E6-AP antibody (kindly provided by Martin Scheffner) for 30 min on ice, followed by adsorption of the immune complexes onto Protein A-Sepharose beads (see Figure 53A for experimental scheme). The supernatant fractions were then used as *in vitro* translated proteins depleted of E6-AP. Immunodepletion with a pre-immune antibody was included as a negative control. To assess the effectiveness of the depletion, we

A.

P-1 ERWWEGVFYELLGLTE **cont-1** FERDEGVLWLLWGYEE

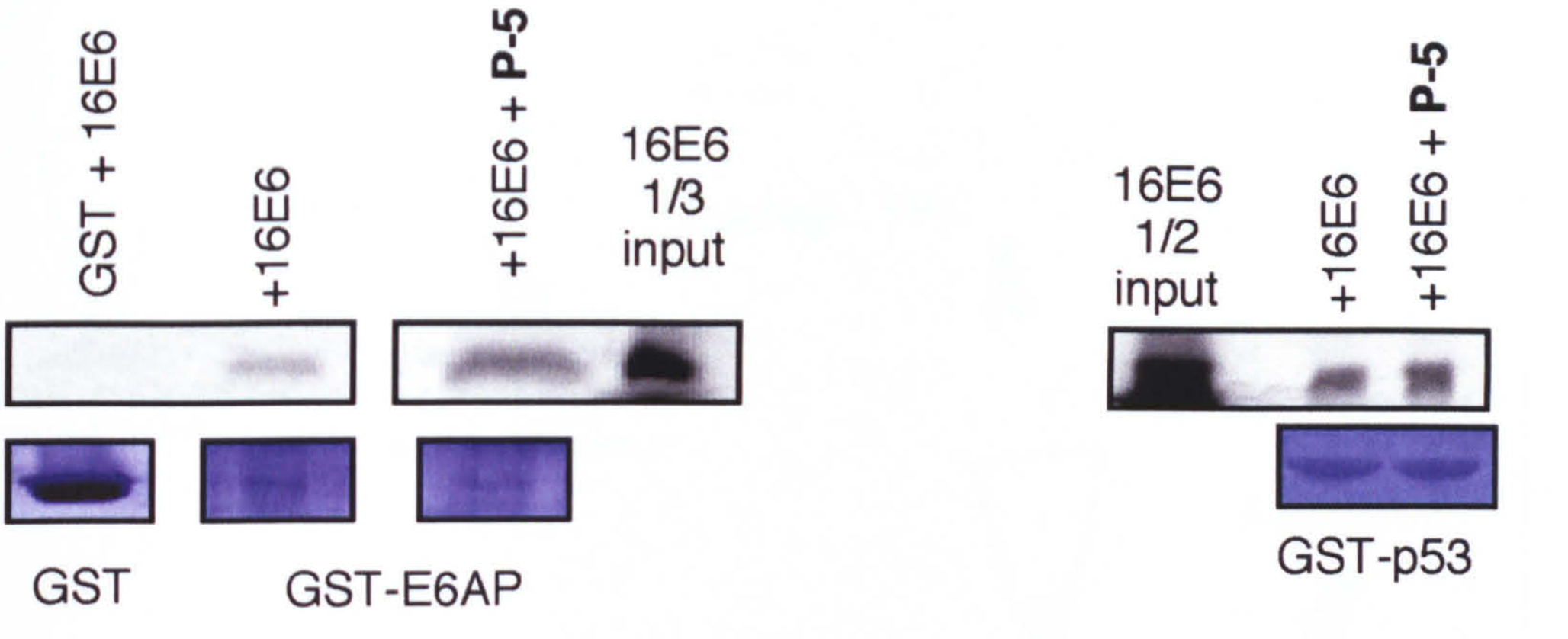
P-5 ERWWEGVFYLLWGYEE

B.

Figure 52. Peptide P-5 retains the ability to block E6-mediated degradation of p53.

(A) Sequence alignment of the synthetic peptides P-1, cont-1 and P-5. (B) P-5 retains binding to HPV-16 and HPV-18 E6. Radiolabelled HPV-16 and HPV-18 E6 were pre-incubated for 20 minutes at room temperature with or without the biotinylated peptide P-5. Proteins bound to the biotinylated peptide were recovered by the addition of avidin-agarose beads. Following extensive washing, bound proteins were detected by SDS-PAGE and autoradiography. Protein inputs for the assay are also shown.

C.



D.

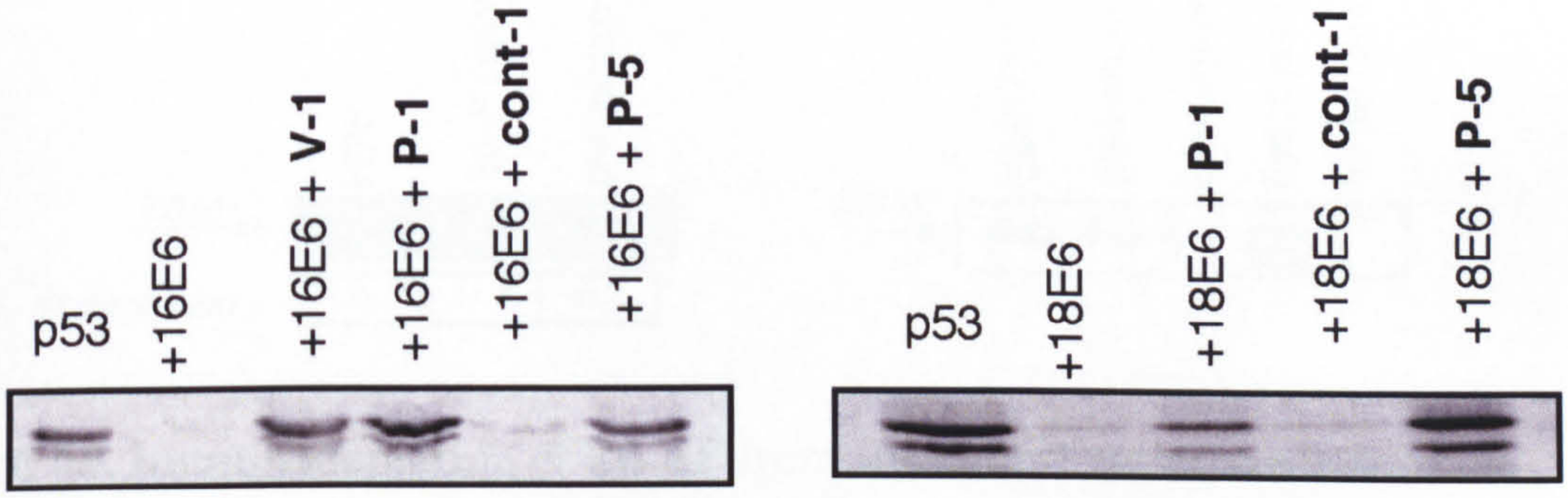
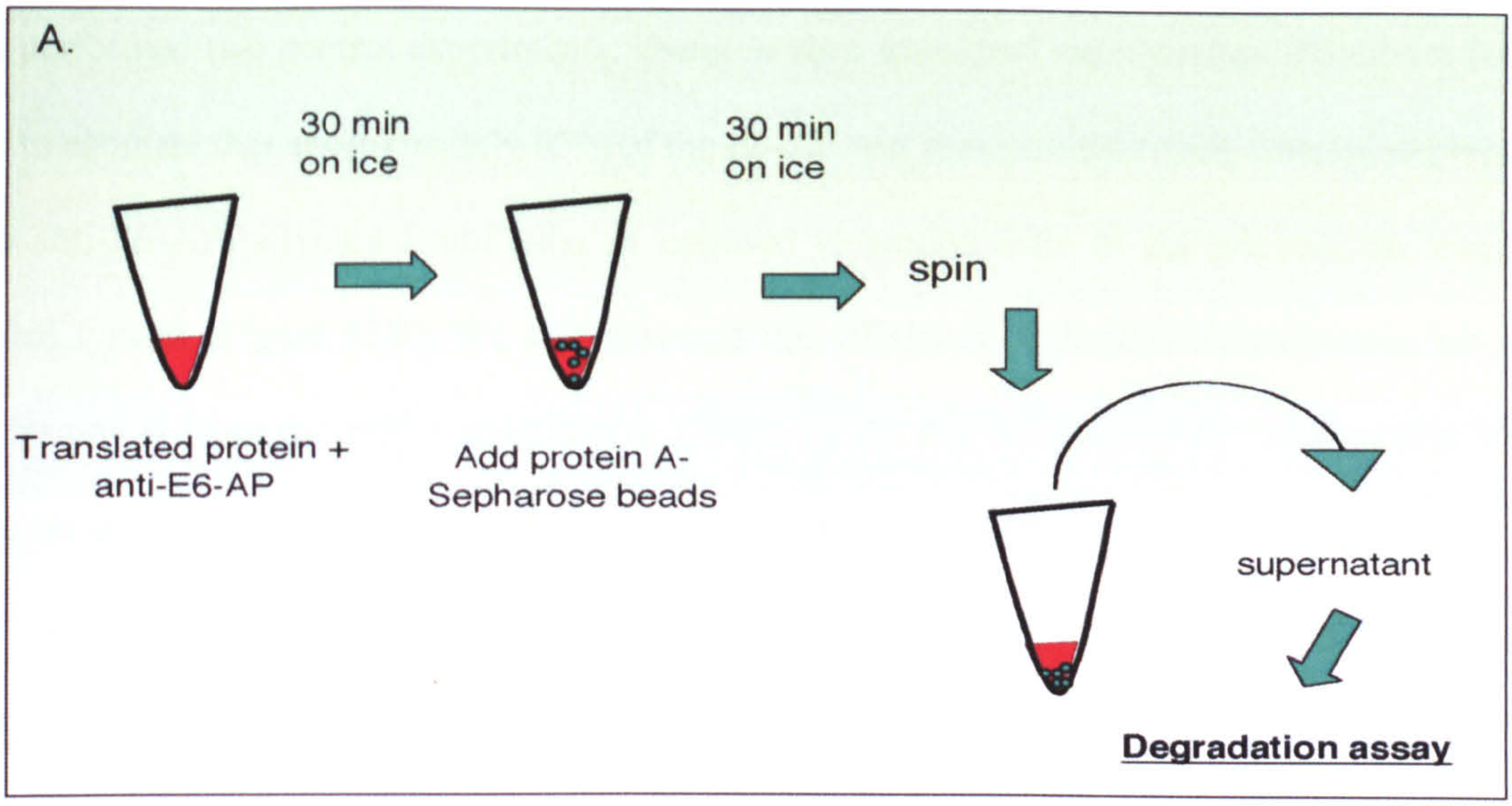
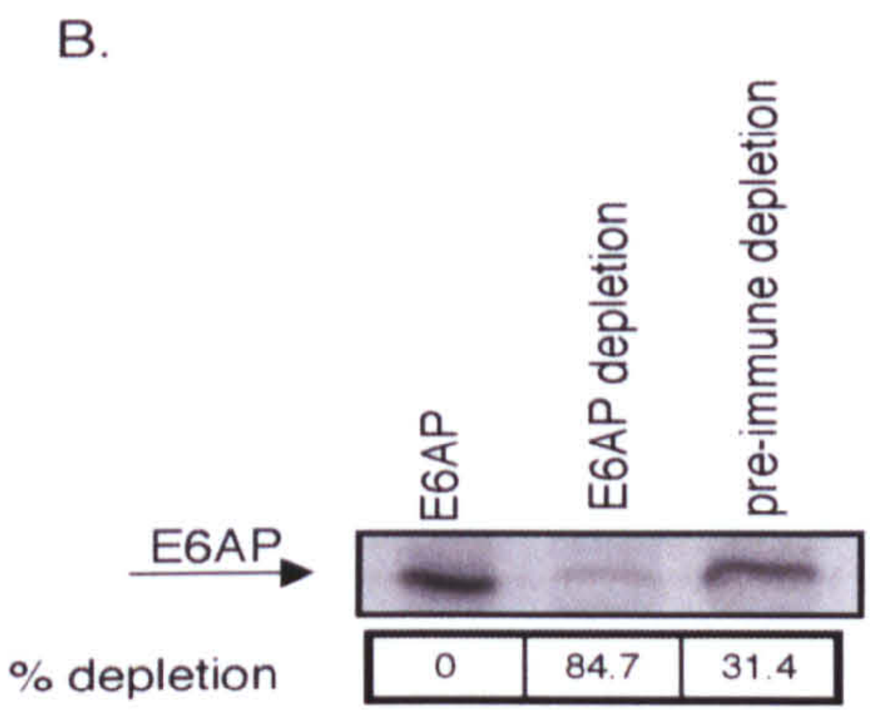


Figure 52. Peptide P-5 retains the ability to block E6-mediated degradation of p53.

(C) A binding assay between GST-E6-AP plus *in vitro* translated HPV-16 E6 and GST-p53 plus *in vitro* translated HPV-16 E6 in the presence and absence of peptide P-5. As can be seen, peptide P-5 has no significant effect on E6 binding to either E6-AP or p53. (D). HPV-16 E6-mediated degradation of p53 is inhibited by peptide P-5. *In vitro* translated p53 was incubated in the presence of either *in vitro* translated HPV-16 or HPV-18 E6 alone or with the peptides (1mM), as indicated, for 30 minutes. The presence of p53 was determined by immunoprecipitation followed by SDS-PAGE and autoradiography. The representative results of three experiments are shown.





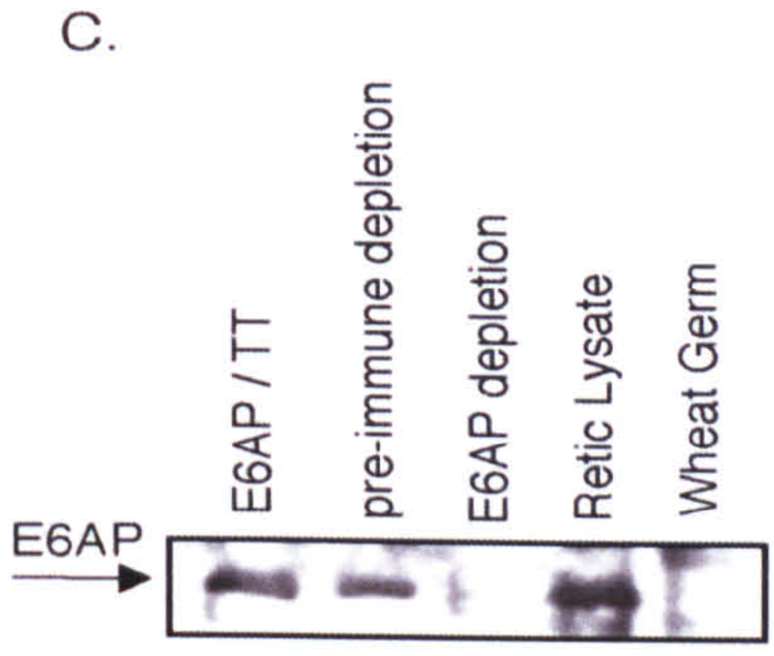


Figure 53. Immunodepletion of E6-AP from the rabbit reticulocyte extract. (A) A schematic representation of the E6-AP immunodepletion assay. (B) Rabbit reticulocyte lysate containing *in vitro* translated E6-AP was depleted by immunoprecipitation with either anti-E6-AP antibody (1 μl) or with pre-immune antibody (1 μl). After depletion, residual E6-AP protein was detected by autoradiography followed by quantification on a Cyclone (Packard). The numbers below each lane show the percentage of E6-AP that is removed. (C) As a second assessment of the efficiency with which endogenous E6-AP is removed from the rabbit reticulocyte lysate, 10 μl was immunodepleted with either anti-E6-AP antibody (1 μl) or pre-immune antibody (1 μl). After depletion, residual E6-AP protein was detected by western blot analysis using rabbit polyclonal anti-E6-AP antibody. Reticulocyte lysate and wheat germ extracts were included as positive and negative controls, respectively. The representative results of at least three experiments are shown.

first performed two control experiments. Using *in vitro* translated radiolabelled E6-AP we were able to estimate that approximately 85% of the E6-AP was removed following immunodepletion with anti-E6-AP polyclonal antibody, as opposed to around 30% in the pre-immune treated control lysates (Figure 53B). We also assessed the efficiency with which endogenous E6-AP was removed from the rabbit reticulocyte lysate. To do this an anti-E6-AP western blot was done on wheat germ extract and on untreated, pre-immune-depleted and E6-AP-depleted rabbit reticulocyte extract. As shown in Figure 53C, no E6-AP was detected in wheat germ extract in comparison with the rabbit reticulocyte extract, in which a strong band was detected at 100 kDa: this is in agreement with previously published data (Huibregtse et al., 1991). Immunodepletion with anti-E6-AP antibody effectively removed most of the E6-AP protein from the rabbit reticulocyte lysate (Figure 53C), whereas, in contrast, the control immunodepletion with pre-immune sera had little effect. Thus, these data show that the polyclonal anti-E6-AP antibody raised against human E6-AP efficiently recognises the rabbit homologue in the rabbit reticulocyte system that we used. This is consistent with the very high degree of conservation in the E6-AP protein found between species, with over 99% homology between human and mouse E6-AP. Furthermore, one round of immunodepletion was sufficient to remove most of the E6-AP protein present within the lysate.

Since it has been demonstrated that the E6-mediated degradation of p53 is E6-AP dependent, we used this to test the effectiveness of our immunodepletion. *In vitro* translated p53 was immunodepleted of E6-AP and then incubated with similarly immunodepleted HPV-16 or HPV-18 E6 for 30 minutes at 30°C. The remaining p53 protein was then immunoprecipitated with rabbit polyclonal C4 antibody and the results obtained are shown in Figure 54. As can be seen, p53 is effectively degraded by E6 over the course of the assay in the sample immunodepleted with the pre-immune antibody (negative control). However, E6-mediated degradation of p53 was blocked in the E6-AP depleted samples, and this agrees with previous publications (Scheffner et al., 1993; Huibregtse et al., 1991, 1993b).

Having confirmed that, by using the E6-AP immunodepletion procedure, we can efficiently

block E6-mediated degradation of p53, we were then interested in investigating the role of E6-AP in the E6-mediated degradation of Dlg and the MAGI family of proteins. Lysates containing radiolabelled E6, Dlg and the MAGI proteins were treated as described above, and degradation assays were then performed. We first investigated the effect of E6-AP immunodepletion on the E6-mediated degradation of Dlg. The assays were performed as for p53, except that the degradation assay requires 2 hrs at 30°C. After incubation the amount of residual Dlg was assessed by immunoprecipitation with rabbit polyclonal anti-Dlg antibody (Mantovani et al., 2001). The results obtained are shown in Figure 55A. As can be seen, in samples immunodepleted of E6-AP, there was the same level of E6-mediated degradation of Dlg as that observed in samples immunodepleted with pre-immune antibody, demonstrating that E6-induced degradation of Dlg is indeed E6-AP independent. We then proceeded to investigate the requirement for E6-AP in E6-mediated degradation of MAGI-1, MAGI-2, and MAGI-3. The MAGIs, plus HPV-16 and HPV-18 E6, were translated *in vitro* in rabbit reticulocyte lysate, immunodepleted as before, and incubated at 30°C for 60 min. The remaining proteins were immunoprecipitated by using rabbit polyclonal anti-WW antibody (Thomas et al., 2002) and analysed by SDS-PAGE. The results obtained are shown in Figure 55 (panels B, C and D). As can be seen, MAGI-1 is degraded to the same extent by HPV-16 and HPV-18 E6 over the course of the assay in both the E6-AP and pre-immune depleted extract (Figure 55B). The same is also true for MAGI-2 and MAGI-3, which are efficiently degraded in E6-AP depleted extract by both HPV-16 and HPV-18 E6 (Figure 55C and D, respectively). These results demonstrate, that whilst E6-AP is required for E6-induced degradation of p53, it does not appear to be required for E6-induced degradation of either Dlg or the MAGI family of proteins.

It was always possible, however, that the Dlg and MAGI proteins were not degraded through the ubiquitin-proteasome pathway in these *in vitro* assays. To test this possibility further, we performed an *in vitro* degradation assay, as described above, with the proteins translated alone or in the presence of either the proteasome inhibitor N-CBZ-Leu-Leu-Leu-al (CBZ) or DMSO as a control. As shown in Figure 56, all the PDZ domain-containing proteins used in this study

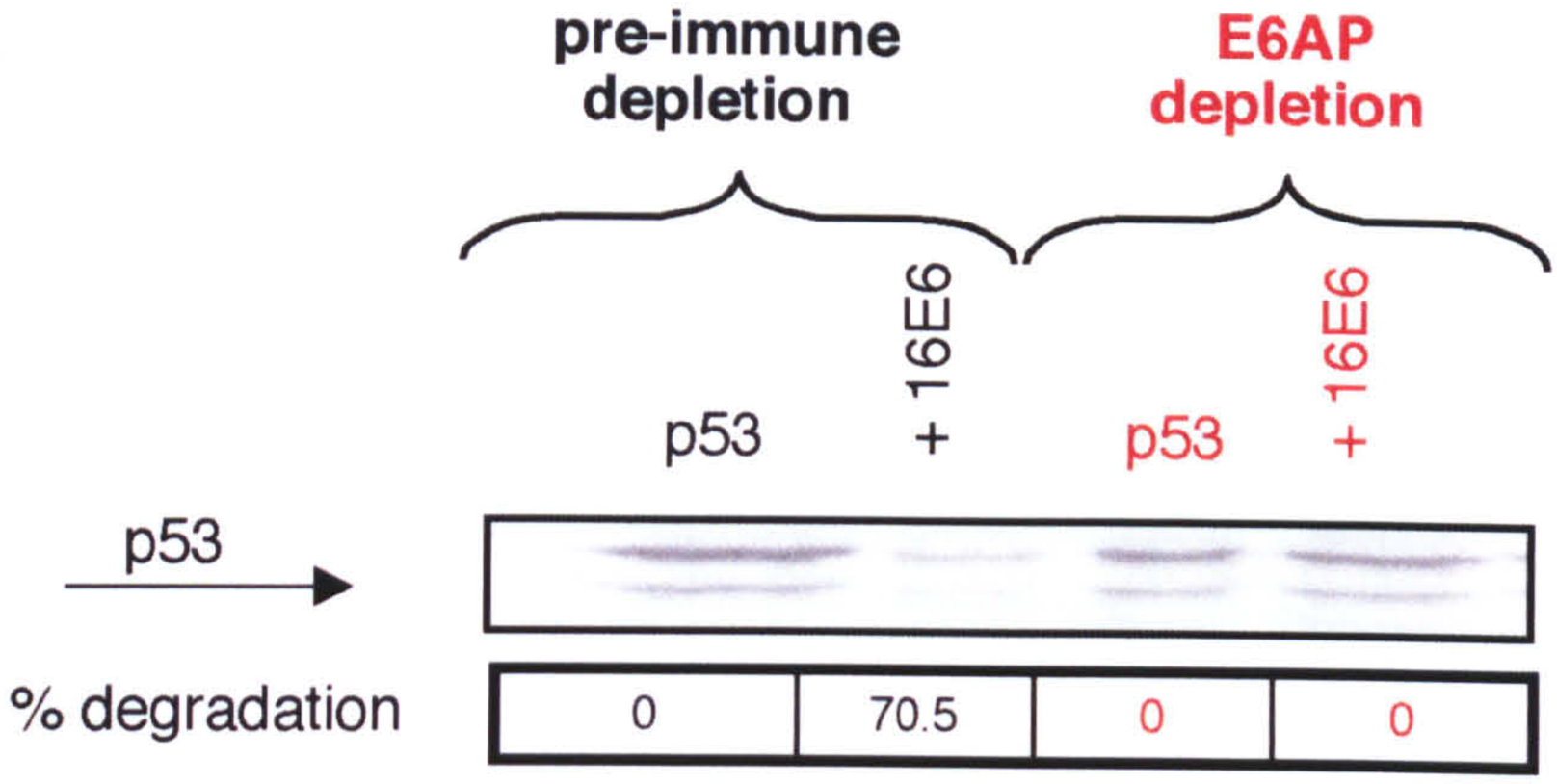
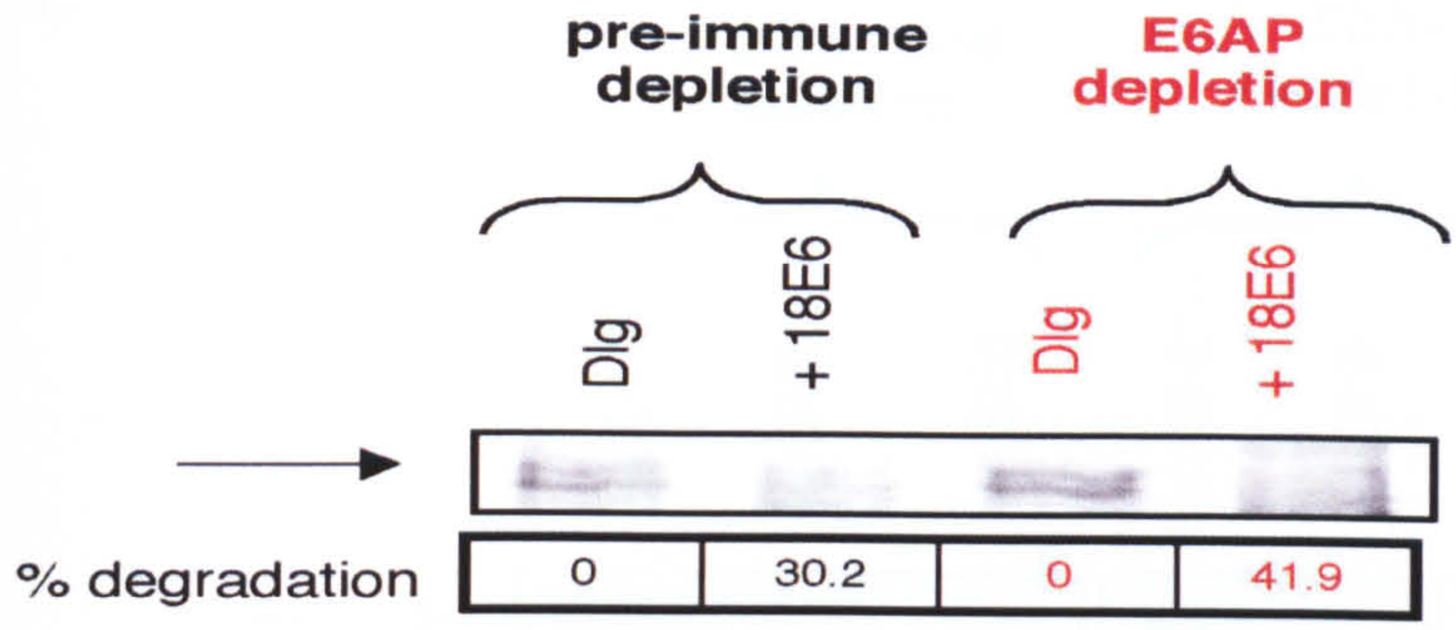


Figure 54. E6-AP immunodepletion blocks the E6-mediated degradation of p53.

p53 and HPV-16 and HPV-18 E6 were translated *in vitro* and depleted with anti-E6-AP or pre-immune antibody. Depleted samples were used in degradation assays, as indicated, and the remaining p53 protein was visualised by immunoprecipitation, followed by SDS-PAGE and autoradiography. The numbers below each lane show the percentage of input p53 that is degraded. Representative results of at least three experiments are shown.

A.



B.

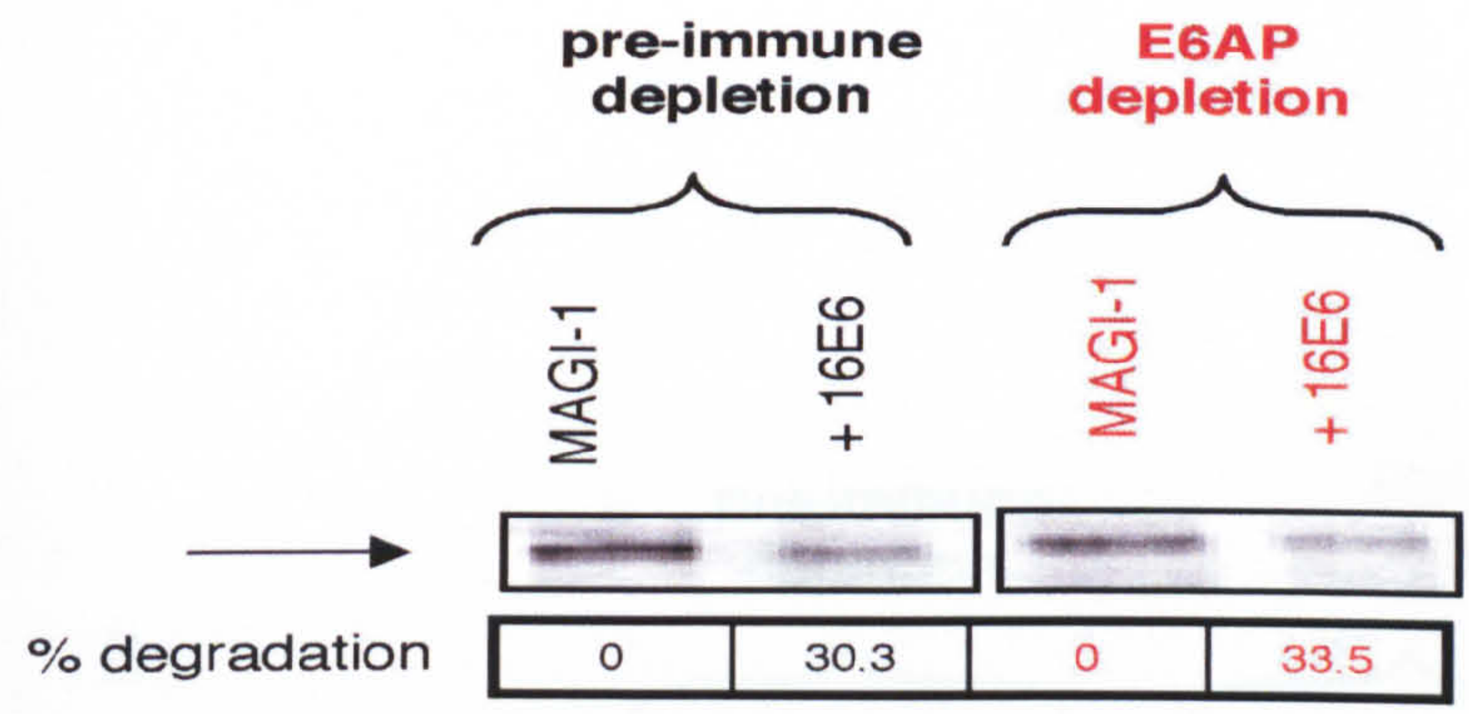


Figure 55. E6-mediated degradation of the PDZ domain-containing proteins, Dlg and MAGI-1, -2 and -3, is E6-AP independent.
In vitro translated E6, Dlg, and the MAGI proteins were subjected to immunodepletion with either anti-E6-AP antibody or a rabbit pre-immune serum. The proteins were then mixed in the combinations indicated and incubated at 30°C for 2 hours for Dlg (panel A) or 1 hour for the MAGIs (panel B). The remaining target proteins were ascertained by immunoprecipitation followed by SDS-PAGE and autoradiography. Arrows indicate the positions of the Dlg and MAGI proteins, and the numbers below each panel show the percentage of input protein that is degraded. Representative results of at least three experiments are shown.

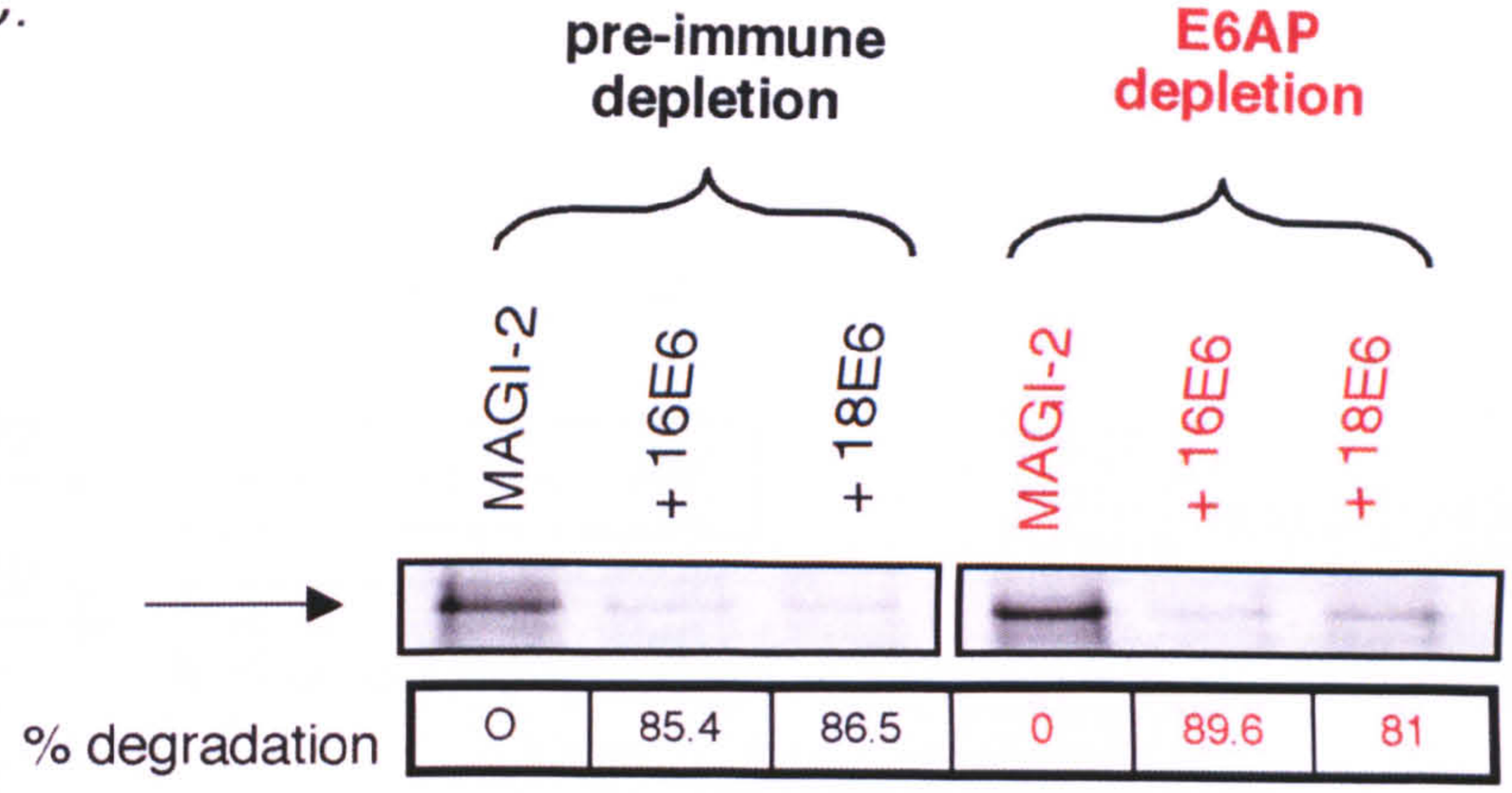


Figure 55. E6-mediated degradation of the PDZ domain-containing proteins, Dlg and MAGI-1, -2 and -3, is E6-AP independent.
In vitro translated E6 and the MAGI proteins were subjected to immunodepletion with either anti-E6-AP antibody or a rabbit pre-immune serum. The proteins were then mixed in the combinations indicated and incubated at 30°C for 1 hour (panels C-D). The remaining target proteins were ascertained by immunoprecipitation followed by SDS-PAGE and autoradiography. Arrows indicate the positions of the MAGI proteins, and the numbers below each panel show the percentage of input protein that is degraded. Representative results of at least three experiments are shown.

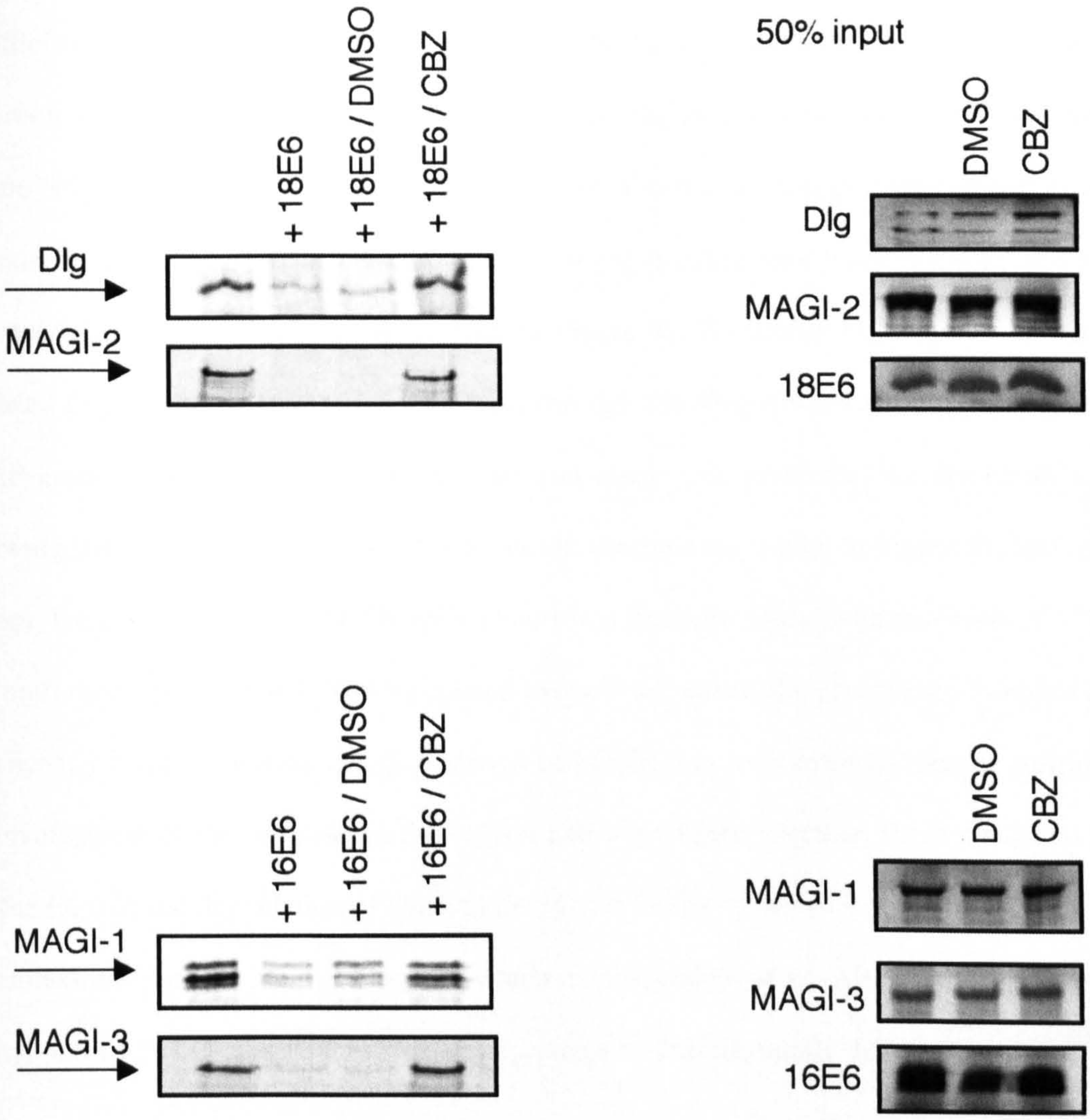
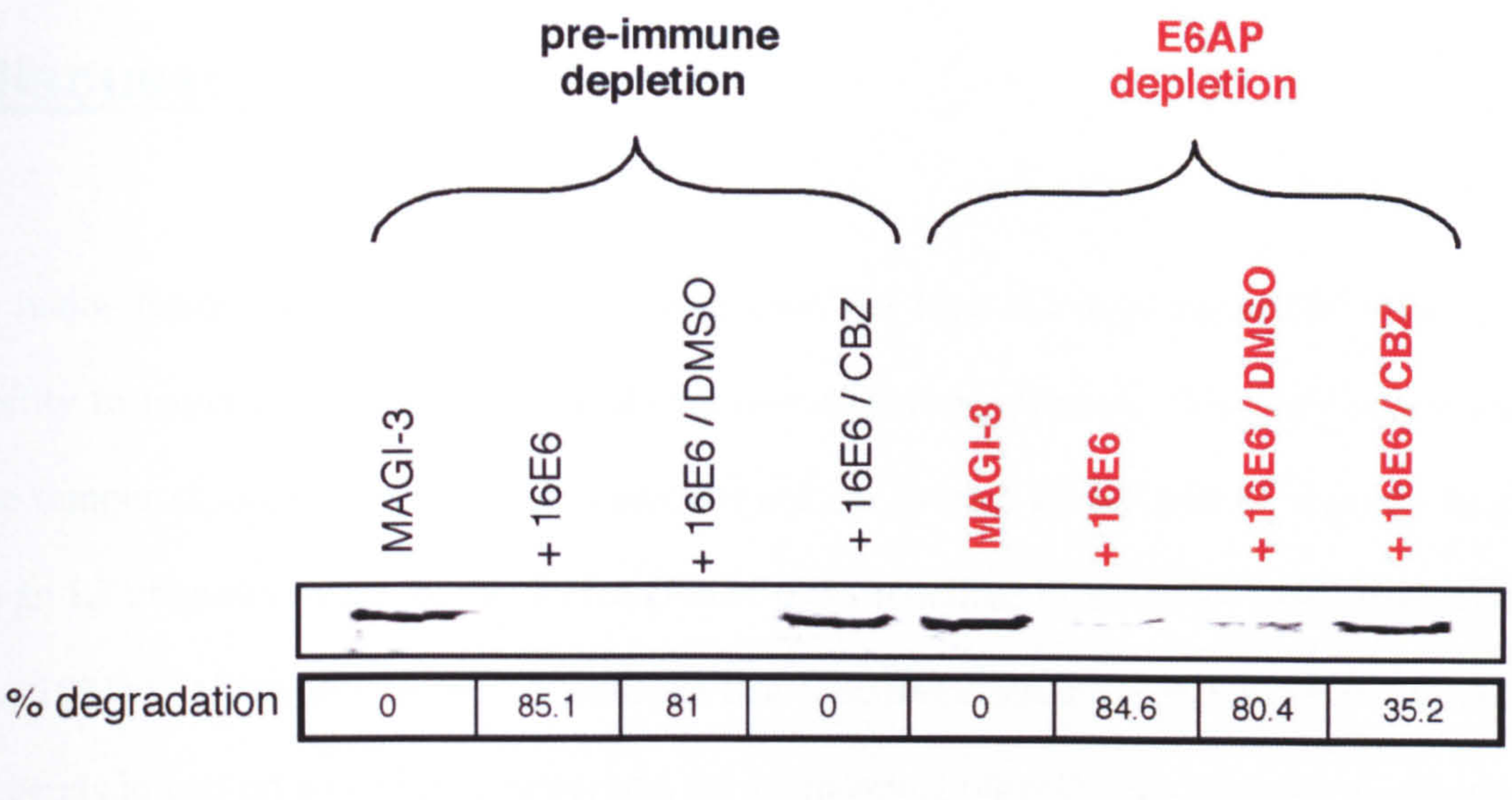


Figure 56. High-risk E6s induce the degradation of Dlg and MAGI proteins via the proteasome.

Dlg, MAGI and E6 proteins were translated *in vitro* using rabbit reticulocyte lysate in the presence or absence of either DMSO or 1mM CBZ. The proteins were then mixed in the combinations indicated and incubated at 30°C for 2 hours for Dlg or 1 hour for the MAGIs. The remaining levels of the target proteins were ascertained by immunoprecipitation followed by SDS-PAGE and autoradiography. Arrows indicate the positions of Dlg and MAGI proteins. The right panels show inputs of proteins used in each assay. Representative results of at least three experiments are shown.

are efficiently degraded in the presence of either HPV-16 or HPV-18 E6. However, proteasome inhibition with CBZ results in a significant increase in the steady-state levels of Dlg and MAGI (Figure 56), indicating that the proteasome is involved in the E6-mediated degradation of these proteins in the *in vitro* system. Translation levels of the proteins used here are not altered in the presence of DMSO or CBZ, as can be seen in Figure 56. To further investigate whether E6-mediated degradation of target proteins occurs through the ubiqutin-proteasome pathway in the E6-AP-immunodepleted extracts, the degradation assay was performed as described above, following immunodepletion of E6-AP. The results obtained are shown in Figure 57, and as can be seen, the presence of HPV-16 E6 again results in a dramatic decrease in the levels of MAGI-3 in both mock depleted and E6-AP depleted extracts. However, the proteasome inhibitor CBZ significantly inhibits E6-mediated degradation of MAGI-3 in both extracts, thereby confirming the involvement of the proteasome proteolytic pathway. Taken together, these results confirm that the E6-induced degradation of Dlg and the MAGI family of proteins occurs *in vitro* through the proteasome pathway, but in a manner which is independent of E6-AP. This further supports our hypothesis that E6 targets these different proteins by fundamentally different mechanisms.



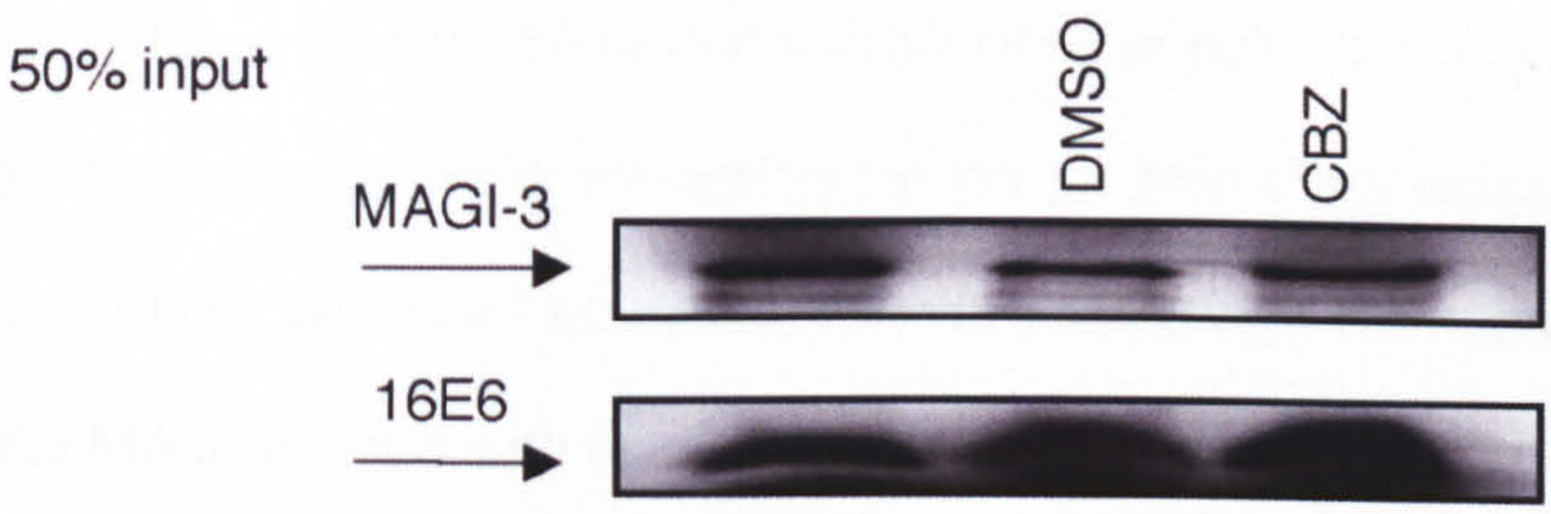


Figure 57. In E6-AP immunodepleted extracts, HPV-16 E6 still targets MAGI-3 for proteasome mediated degradation.

^{35}S-labelled MAGI-3 and HPV-16 E6 proteins were translated *in vitro* using rabbit reticulocyte lysate in the presence or absence of either DMSO or 1mM CBZ. After pre-immune or E6-AP immunodepletion, the MAGI-3 and E6 proteins were mixed and incubated at 30°C for 1 hour. The remaining MAGI-3 was detected by immunoprecipitation and autoradiography. The numbers below each panel show the percentage of input MAGI-3 that is degraded. The lower panel shows the input of MAGI-3 and E6 proteins used in each assay. Representative results of at least three experiments are shown.

Discussion

A major feature of the E6 proteins derived from the high-risk papillomavirus types is their ability to target cellular proteins for ubiquitin-mediated degradation. This was first shown for the tumour suppressor protein p53, where the cellular protein E6-AP and E6 together function as an E3 ubiquitin ligase for the degradation of p53 (Scheffner et al., 1990, 1993; Huibregtse et al., 1993b). Although it is well established that transformation by high-risk HPV E6 proteins depends in part on an ability to target p53 for degradation, other E6 functions are also known to be important (Song et al., 1999). More recently, a number of the MAGUK proteins have been described as targets for an E6 protein-dependent degradation through the ubiquitin-proteasome pathway, including Dlg (Gardiol et al., 1999) and the MAGI proteins (Glaunsinger et al., 2000; Thomas et al., 2001b, 2002). There is mounting evidence that the degradation of MAGUK proteins by E6 is different from the E6-mediated degradation of p53 (Pim et al., 2000; Thomas et al., 2001b, 2002), but the molecular mechanism has not yet been characterized. In an attempt to elucidate this we have performed an extensive *in vitro* analysis of the interactions between p53, Dlg, and the MAGI proteins with the E6 proteins of HPV types 16 and 18 in the presence of synthetic peptides which specifically interact with the HPV E6 protein (Elston et al., 1998; Sterlinko Grm et al., 2004). In a series of assays, we show conclusively that the E6 proteins target these cellular substrates through different pathways.

The region of E6-AP required for its stable association with HPV-16 E6 was originally mapped to 18 amino acids in the central portion of the molecule, and a peptide composed of the 18-amino-acid sequence was able to compete for the binding of HPV-16 E6 to E6-AP (Huibregtse et al., 1993b). Previously, Elston et al. (1998) identified peptides that were homologous to the 18-amino acid E6 binding domain within E6-AP. Three out four of the selected peptides contained alpha-helical ELLG motifs, and the best of these, peptide 1 (P-1) was used as a template for the generation of a second "E6-specific" peptide library, in order to identify ELLG-containing peptides that associate with E6 more efficiently (Sterlinko Grm et al., 2004). The

peptide V-1 was isolated in John Doorbar's laboratory from this second screen and identified as being the most improved peptide, with the highest binding efficiency for E6 (Sterlinko Grm et al., 2004).

Knowing that one of the most important features of high-risk E6 proteins is the degradation of their cellular targets, we were naturally interested in investigating the effects of the two peptides, P-1 and V-1, upon this activity of E6. Since these peptides are based on the prototype E6-AP interaction motif we first analysed their effects upon the E6/E6-AP interaction. The results demonstrate that both V-1 and P-1 efficiently inhibit the interaction between E6 and E6-AP, and they also show that V-1 is the more effective inhibitor of the interaction, as would be expected given its apparent increase in affinity for E6. However, neither peptides had any effect upon the ability of E6 to bind p53, regardless of whether the assays were performed in the presence (*in vitro* translated in reticulocyte lysate) or absence (*in vitro* translated in wheat germ extract) of endogenous E6-AP, thus confirming that E6-AP is not required for E6's association with p53. These results contradicted studies that had suggested E6-AP was required for p53 binding (Huibregtse et al., 1993a), whilst being in agreement with other studies (Lechner & Laimins, 1994; Li & Coffino, 1996). The reasons for this could lie in the different experimental conditions between different studies (Huibregtse et al., 1993a), but the fact that others also observed a strong interaction between E6 and p53 in the absence of E6-AP (Lechner & Laimins, 1994; Li & Coffino, 1996) strongly suggest that the contribution of E6-AP to the interaction between E6 and p53 is, at best, minimal.

We next investigated whether the binding of these peptides to E6 would inhibit the degradation of p53. As expected, pre-incubation of either V-1 or P-1 with HPV-16 E6 markedly inhibits its ability to induce p53 degradation. Since the interaction between HPV-16 E6 and p53 is not disrupted by the peptides, this would rule out the possibility that the block to degradation is a reflection of the inhibition of E6/p53 binding. Since HPV-16 E6 has consistently been shown to bind and degrade p53 more effectively than HPV-18 E6 (Werness et al., 1990; Scheffner et al., 1990), we proceeded to determine whether this is reflected in the abilities of the peptides to

inhibit the degradation of p53 by the two E6 proteins, but found no difference in the efficiency with which the peptides blocked their activities. Having shown that V-1 and P-1 inhibit the degradation of the p53 protein, we then analysed which was more efficient and found that peptide P-1 is more effective than V-1. This was particularly interesting since V-1 was selected for being the stronger binder, and in agreement with this we found that pre-incubation of V-1 with HPV-16 E6 protein inhibits its ability to complex with E6-AP more strongly than pre-incubation with P-1. Taken together, these results indicate that there is no correlation between the inhibition of E6's binding to E6-AP and inhibition of E6-mediated degradation of p53, suggesting that degradation of p53 by E6 does not simply involve recruitment of E6-AP but that other cellular proteins may also be involved. Previous studies have shown that the wild type E6-AP peptide (P-E6-AP), containing the ELLG motif, can inhibit the E6/E6-AP interaction at high concentrations (Huibregtse et al., 1993a; Elston et al., 1998). However, we never observed any inhibitory effect of this peptide, either upon the E6 association with E6-AP or with p53, nor in any of our *in vitro* degradation assays. Only the higher affinity peptides that were selected were effective in inhibition. These observations have a number of important implications: first, they imply that the basal interaction between E6 and E6-AP may be rather weak. Secondly, they also raise the prospect that there may be other ubiquitin protein ligases binding to E6 with a higher affinity at this same region of the E6 protein, and whose interaction can only be blocked by the high affinity peptides. At this stage, we cannot rule out the possibility that the peptides affect the E6-AP interaction with the E2 conjugating enzymes, UbcH5, 6, 7 and 8 (Nuber et al., 1996; Huang et al., 1999) or that they interfere with the formation of E6-AP-ubiquitin thiolester intermediates. However, since our data suggest that Dlg and MAGI degradation is E6-AP independent (see below), this seems unlikely.

As a means of understanding the basis for the improved efficiency of the P-1 peptide, we also made P-5, which contains the amino terminal half of P-1 and the carboxy terminal half of the jumbled peptide cont-1. The P-5 does not have the ELLG motif, previously shown to be important for the E6/E6-AP interaction (Huibregtse et al., 1993b; Elston et al., 1998; Be et al.,

2001), and, as expected, the E6/E6-AP and E6/p53 interactions were unaffected by P-5. However, when assayed for its effects on E6-induced degradation of p53, P-5 was found to be a potent inhibitor. These studies suggest a far more complex pattern of interactions than the simple recruitment of E6-AP, and strongly support the existence of additional cellular proteins that are involved in the E6-mediated degradation of p53, identification of which is now a high priority.

In an attempt to identify these cellular proteins, we used a Blast search for short sequences, and discovered that 6 out of 8 amino acid residues in the amino terminal half of P-5 were identical to a sequence in the amino terminal region of the CIN85 protein (Figure 58). CIN85 (Cbl-interacting protein of 85 kDa) is a broadly expressed multi-adaptor protein containing three Src homology (SH3) domains at the amino terminus, a proline-rich region and a coiled-coil domain in the carboxy terminus (Take et al., 2000; Dikic, 2002). This multi-domain protein binds to the adaptor proteins Grb2, Crk, and p130Cas, and can form larger protein complexes after oligomerization mediated by the coiled-coil domain (Watanabe et al., 2000). Furthermore, it was shown recently that CIN85 also binds to the distal carboxy terminus of the Cbl/Cbl-b ubiquitin ligase, which is responsible for mediating the ubiquitination and subsequent lysosomal degradation of many receptor tyrosine kinases (RTKs), among them epidermal growth factor (EGF) receptors (Thien & Langdon, 2001; Dikic et al., 2003). These observations raise the possibility that CIN85 might represent the missing link in the E6/E6-AP-mediated degradation of p53. To address this hypothesis, we looked at whether CIN85 could interact directly with the E6-AP ubiquitin ligase or E6 *in vitro*, or affect E6-induced degradation of p53. To date, all assays have been negative, suggesting that CIN85 is not the adaptor protein involved in the E6-mediated degradation of p53. However, additional experiments should be done to exclude completely the possibility of CIN85 being involved in the E6-mediated degradation of p53, such as the use of dominant negative mutants or siRNA against CIN85, and future studies will address this issue.

To further investigate the role of the ELLG motif and E6-AP upon the degradation of other

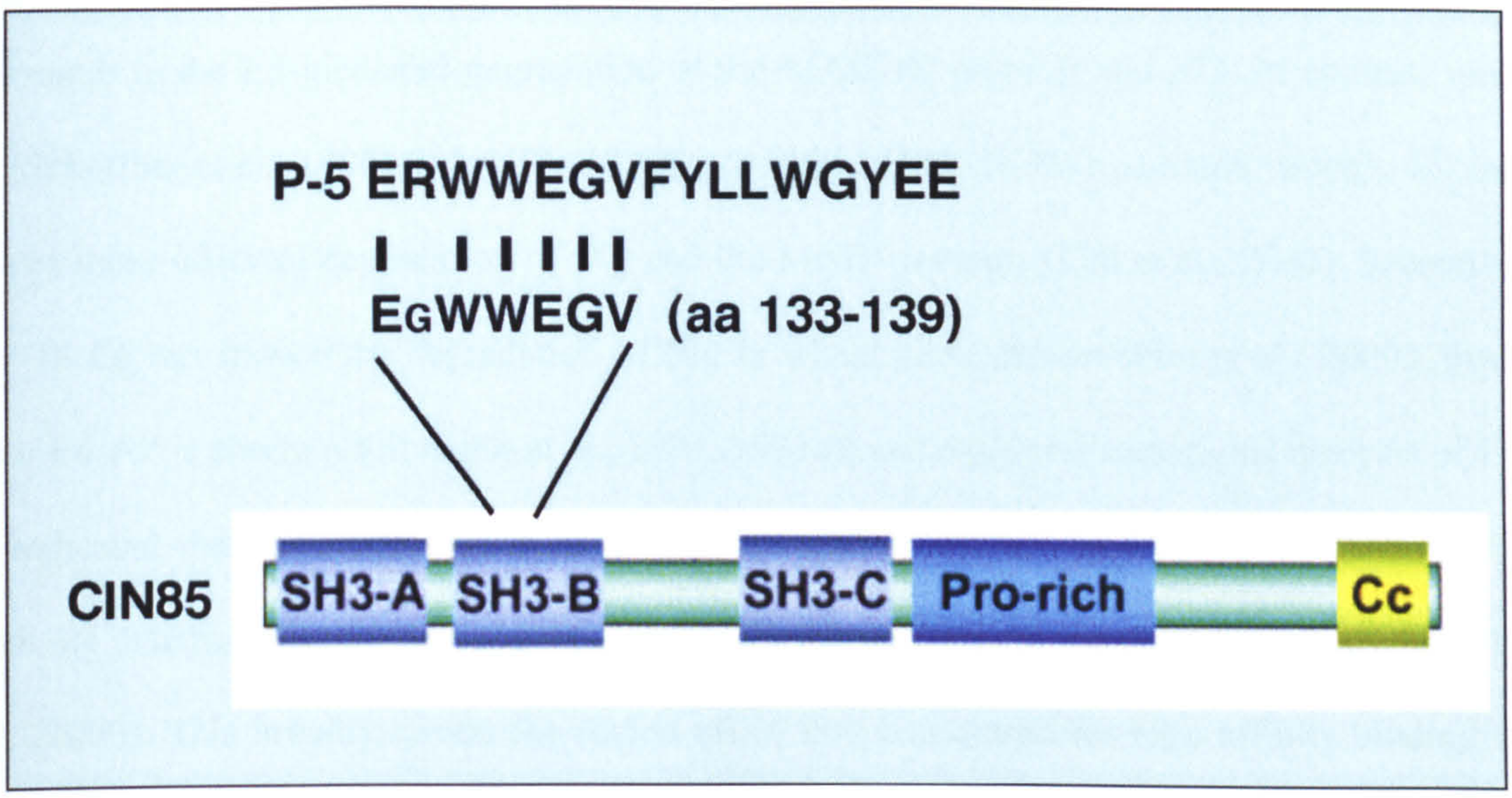


Figure 58. Schematic representation of the domain structure of CIN85. CIN85 contain three N-terminal SH3 domains, a centrally located proline-rich motif and a C-terminal coiled-coil domain. They were isolated in human, rat and mouse, while there are no orthologues found in *D. melanogaster*, *C. elegans* or yeast cells (Modified from Dikic, 2002). Note the region of sequence identity found between the SH3-B domain (aa 133-139) of CIN85 and the amino terminal half of synthetic peptide, P-5.

targets of E6, the study was extended to the MAGUK proteins Dlg, MAGI-1, MAGI-2 and MAGI-3, which are also targets for E6 induced degradation (Gardiol et al., 1999; Glausinger et al., 2000; Thomas et al., 2002). Previous studies have shown that there are significant differences in the E6-mediated degradation of the MAGUK proteins and p53. In contrast with p53 (Scheffner et al., 1990), it is HPV-18 E6 rather than HPV-16 E6 that binds strongly to, and induces more efficient degradation of Dlg and the MAGI proteins (Pim et al., 2000). Secondly, HPV-18 E6 can induce the degradation of Dlg in wheat germ extract (Pim et al., 2000), from which E6-AP is absent (Huibregtse et al., 1991, 1993b); and extensive mutational analysis of E6 has indicated that sequences which lie within the amino terminal half of the E6 protein and which are essential for p53 degradation, are largely dispensable for the degradation of Dlg (Pim et al., 2000). This broadly covers the region of E6 that is required for high affinity binding to E6-AP (Li and Coffino, 1996). Lastly, low-risk HPV E6 proteins, which do not interact with E6-AP, can degrade Dlg when provided with a PDZ-binding consensus sequence (Pim et al., 2000). All these facts indicate that E6-AP is probably not involved in the degradation of Dlg. Surprisingly, both V-1 and P-1 inhibited the HPV-18 E6-mediated *in vitro* degradation of Dlg in the same way as they inhibited the degradation of p53 protein. These results enable us to conclude that the peptides, despite their homology with E6-AP, may not only inhibit the interaction between E6 and E6-AP, but may also prevent interaction between E6 proteins with, as yet unknown, adaptor protein(s) or ligase(s), which are required for E6 activity.

In contrast with Dlg, the effects of the peptides upon HPV-18 E6-induced degradation of the MAGI-1, MAGI-2 and MAGI-3 proteins was somehow different. The results demonstrated that P-1 efficiently inhibited E6-mediated degradation of MAGI-1, whilst V-1 was completely inactive. This contrasts markedly with the results obtained with p53 where V-1 is a potent inhibitor of E6-induced degradation. Therefore, these results clearly show that the E6-induced degradation of MAGI-1 is different from E6-induced degradation of p53 and Dlg. Interestingly, previous studies have observed similar differences. For example, mutational analysis of E6 identified mutants which were defective for degradation of one target, but still active for the

other, and at the same time they also suggested that the E6-induced degradation of Dlg and MAGI-1 occurred through similar mechanisms (Thomas et al., 2001b; Pim et al., 2000). However, while the regions of E6 required for degradation might be similar, the marked differences in kinetics is more supportive of the conclusions gained from the peptide inhibition experiments. Thus MAGI-1 is highly susceptible to E6-induced degradation, with complete clearance of protein in 30 min (Glaunsinger et al., 2000); in contrast, complete clearance of Dlg requires at least 2 hrs (Gardiol et al., 1999).

One of the most interesting findings in this part of the study was that P-1 does not appear to inhibit E6-mediated degradation of MAGI-2 and MAGI-3. Since there is considerable homology between all three MAGI proteins this was particularly striking. The fact that MAGI proteins are members of the MAGUK-family suggests that these PDZ proteins function to assemble numerous cellular targets into large signaling complexes in cells. Despite the high homology between all three MAGI proteins, they probably do not all share similar binding partners and, consequently, do not have the same functions. In this context, it is tempting to speculate that E6 might use different ubiquitin ligases or adaptor proteins, which are involved in the regulation of the degradation complex, to target MAGI proteins for degradation. In this way, they could disrupt or prevent the formation of various different signaling complexes, that are involved in a number of pathways leading to cell survival and proliferation.

Having shown that a panel of inhibitory peptides exerted different effects on the ability of HPV E6 to direct the degradation of a number of its substrates, we were obviously interested in determining if these differences reflect the involvement of the same ubiquitin ligase, E6-AP and different adaptor proteins, or if a second E6-associated ubiquitin ligase exists. To investigate this, E6-AP immunodepletion experiments were done. Removal of E6-AP completely abolishes the ability of E6 to degrade p53, and this is consistent with previous observations (Huibregtse et al., 1991, 1993b; Scheffner et al., 1993). In contrast, E6 can direct the degradation of Dlg and the MAGI proteins in the absence of the E6-AP ubiquitin ligase in an *in vitro* system, an observation which has also been confirmed recently in an *in vivo* system using an E6-AP -/-

Baby Rat Kidney cell line (Massimi P., personal communication). These data suggests the existence of a second E6-associated ubiquitin ligase, identification of which is now a high priority.

From previous studies it is clear that members of the papillomavirus family are important pathogens and equally important as the investigation into the basic mechanisms of HPV pathogenesis is the development of effective therapeutics to treat or prevent HPV-induced disease. The identification of drugs specifically to treat HPV infection has not been highly successful due to the complexities of the HPV life cycle and the limited number of enzymatic activities identified for HPV proteins. Therefore, the ability of "our" synthetic peptides to block E6-induced degradation of cellular proteins known to be important in cellular transformation, suggest that they may have considerable potential in the chemotherapy of HPV-induced disease.

Most of the work described in this section is contained in the following articles:

Sterlinko Grm H, Weber M, Elston R, McIntosh P, Griffin H, Banks L & Doorbar J. Inhibition of E6-induced degradation of its cellular substrates by novel blocking peptides. *Journal of Molecular Biology* 2004; **335**: 971-985.

Sterlinko Grm H & Banks L. Degradation of hDlg and MAGIs by human papillomavirus E6 is E6-AP-independent. *Journal of General Virology* 2004; **85**: 2815-2819.

Future directions

Our finding that there is a complex pattern of protein-protein interaction between different HPV encoded proteins provides new insights into how the virus replicates as well as how breakdown in this process might contribute to malignancy. Although it is well known that the oncogenic E6 proteins interfere with several cellular pathways in order to create a favourable environment for viral replication, this does not appear to be the only role of E6 in the viral life cycle. We have demonstrated that E6 interacts with E2, resulting in changes in the substrate specificities of E6 and the biochemical activities of E2. Moreover, when coexpressed E2 and E6 induce marked changes in the pattern of each other's expression with preferential accumulation in nuclear speckles, known as SFCs. However, important questions still remain. The molecular basis for the localisation of HPV E2/E6 complex at SFCs is unknown at present. Are these active sites of gene expression? If so, what is transcribed, cellular or viral genes? In addition, since SFCs are also ideally suited to play a role in alternative-splice-site selection, we must determine if E2/E6 can modulate alternative splice site use.

One of the most intriguing observations in this study is the interaction between the L2 structural protein, whose expression coincides with keratinocyte differentiation in the upper epithelial layers, and E6, which is expressed in the lower and middle layers of the epithelium. Whether an association between the two proteins occurs in HPV induced lesions is the subject of speculation, since there is no data available on the comparative staining of E6 and L2 *in vivo*. Therefore, comparison of the expression of L2 with respect to surrogate markers for the expression of E6 and E7, such as PCNA in rafts or in HPV induced lesions, is likely to provide important information on the relevance of the E6/L2 association *in vivo*, and this analysis is of high priority. Additional questions also remain. Assuming that L2 recruitment of E6 to PODs is biologically relevant, the effects remain to be determined. Preliminary results show that E6 has no effect on SUMO-1 accumulation in L2-staining PODs, although it is possible that E6 interferes with the sumoylation of L2 or other cellular proteins present in this subcellular compartment. This could be tested by performing a series of sumoylation experiments first

asking if L2 itself is a substrate for sumoylation, as well as determing if L2/E6 stimulate/inhibit the sumoylation of cellular components of PODs (such as PML, Daxx or Sp100). Moreover, there is compelling evidence that Daxx is subjected to a complex pattern of regulation. L2 induces its accumulation in PODs, but when E6 is also present, Daxx is degraded. These events can modulate Daxx stability in a dynamic fashion, depending upon the phase of the viral life cycle. However, additional experimental work will be needed to investigate in further detail the molecular mechanisms of Daxx regulation by L2/E6 and its biological consequences. Important questions still remain. First, the role of L2/Daxx association in the viral life cycle, next the possibility of the interaction between E6 and Daxx, as shown for adenovirus E1B 55K (Zhao et al., 2003), and finally it has to be established whether this is the main function of the L2/E6 complex.

The final part of this thesis describes studies which investigate the ability of E6 binding peptides to block E6 activity. An important question arising from these studies is the role of E6-AP in E6 activity. Future work will use the sequences of the blocking peptides to identify potential adaptor proteins involved in the regulation of E6-mediated degradation of p53. An appealing hypothesis would be that the CIN85 could control the E6-mediated downregulation of p53 as it does for EGF receptor. However, preliminary results would indicate that this hypothesis is not correct, and an alternative adaptor protein seems more likely to be involved. The second set of data obtained with the synthetic peptides demonstrate significant differences in how different PDZ containing-proteins are degraded by E6 oncoproteins and provides strong evidence for the role of a second E6-associated ubiquitin ligase, identification of which is now a high priority. Besides representing useful tools for future structure/function analyses of E6, the peptides identified in this study could also facilitate the design of efficient therapeutic drugs that target the E6 oncoprotein. However, some points remain unresolved: it has not been formally demonstrated that this blocking effect occurs also *in vivo*, therefore the most effective peptide P-1 should be introduced into cells by *in vivo* protein transduction methods using the protein transduction domain (PTD) of HIV-1 encoded Tat protein (Schwarze & Dowdy, 2000). As well

as using these peptides to block E6 activity with regard to its cellular targets, it will also be interesting to assess their activity with respect to the interaction between E6 and its viral partners, E2 and L2. This offers enhanced therapeutic potential, since specific inhibitors of these associations are likely to be less toxic than ones which target viral-cellular protein interactions.

Materials and methods

Plasmids

For GST fusion protein production, HPV-16 E1, HPV-16 E2, HPV-16 and -18 E6, HPV-18 E6*I, HPV-18 E6*I ΔM, HPV-16 E7, E6-AP, p53, Dlg and mutant Dlg-NT fusion proteins have been described previously (Storey et al., 1995; Piccini et al., 1995; Kuhne et al., 2000; Pim et al., 1997, Pim & Banks, 1999; Massimi et al., 1996; Huibregtse et al., 1993a; Thomas et al., 1995; Lee et al., 1997). GST-16 E4 and GST-16 L1 were kindly provided by John Doorbar. GST-11 E2 was kindly provided from Noor Gammoh. His6-16 E2-containing vector was generated in the laboratory by in-frame ligation of the full length HPV-16 E2 into the vector pQE9 containing the 6-histidine-tag (His)6.

The SP64 plasmids containing wild type and mutant HPV-18 E6s, as well as wild type HPV-11 E6, HPV-16 E6 and HPV-16 E7, used for in vitro translation have been described previously (Pim et al., 1994, 2002; Massimi et al., 1996). The wild type HPV-16 E2 was cloned into pcDNA3, and its carboxy and amino terminal halves together with a series of E2 carboxy terminal truncation mutants were also cloned in SP64 and have been described previously (Piccini et al., 1995; Massimi et al., 1999). pGEM HPV-16 L2 was kindly provided by Richard Roden.

The pSP64 plasmid containing p53 used for in vitro transcription and translation have been described previously (Pim et al., 1994). Plasmids for expressing E6-AP, Dlg and MAGI-1, MAGI-2, and MAGI-3 in vitro were available in the laboratory and have been described previously (Pim et al., 1997; Gardiol et al., 1999; Thomas et al., 2001b).

For eukaryotic expression, the HA-tagged HPV-18 E6 construct expressing just the full length protein (NonSplicing mutant) and HA-tagged HPV-18 E6*I construct have been described previously (Guccione et al., 2004b). The HA-18E6 ΔM (Δ28-31) construct was generated using the Genetailor Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer's instructions. HA-tagged HPV-16 E6, HPV-18 E6 and HPV-11 E6 in the GWI expression vector

were kindly provided by Ronald Javier, the HPV-16 E6 Δ123-127 construct was kindly provided by Dennis McCance, and the Flag-tagged wild type HPV-18 E6 and the ΔM mutant protein were kindly provided by Greg Matlashewski.

HA-tagged HPV-16 E7 was available in the laboratory and has previously been described (Guccione et al., 2002), as has pCMV HPV-16E2 (Bouvard et al., 1994b). The plasmid pCGE1BΔE5, expressing the HPV-16 E1 and E2 proteins, as well as the plasmid 16URR::CAT containing the HPV-16 origin of replication have also been described previously (Bouvard et al., 1994b; Piccini et al., 1997). The p6xE2BS-Luc reporter plasmid was kindly provided by Ian Morgan and the GFP-tagged HPV-11E2 was kindly provided by Merilyn Hibma.

The wild type p53 was expressed under the control of the CMV promoter and has been described previously (Taurina & Jenkins, 1993). Flag-tagged p53 was kindly provided by Georgine Faulkner. HA-tagged MAGI-1 and MAGI-2, plus V5-tagged MAGI-3 have also been described previously (Thomas et al., 2001b, 2002).

Plasmids expressing Flag-tagged PML isoforms I-VI in pCIneo have previously been described (Guccione et al., 2004a), as has the pUF3 HPV-16 L2 expression plasmid which was kindly provided by Martin Muller (Leder et al., 2001).

Antibodies

HPV-16 E2. Polyclonal antibodies against HPV-16 E2 (WB: 1:500; IF: 1:200) obtained previously (Bouvard et al.,1994b) were raised against the C-terminal domain of the GST-E2 protein.

HPV-16 L2. Anti-L2 rabbit polyclonal antiserum was generously provided by Martin Muller (IF: 1:2000).

p53. p53 was detected using a pool of the anti-p53 monoclonal antibodies pAb 1801, 1802 and 1803 (Banks et al., 1986). For immunoprecipitations, the C4 antibody was used (Pim et al., 1994), which is a polyclonal rabbit antiserum raised against the carboxyl terminal 14 amino acids of p53.

Dlg. Polyclonal antibodies against Dlg were kindly provided by P. Massimi, and were raised against a GST-Dlg N-terminus fusion protein, which comprises amino acid residues 1-222 of rat Dlg (Lee et al., 1997) as described previously (Mantovani et al., 2001).

MAGIs. MAGI-1, MAGI-2 and MAGI-3 were immunoprecipitated after the *in vitro* degradation assays using a rabbit polyclonal anti-WW antibody, raised against a GST fusion protein with the WW domains of MAGI-1, as previously described (Thomas et al., 2002).

E6-AP. Anti-E6-AP rabbit polyclonal antiserum was generously provided by Martin Scheffner.

Commercial antibodies were obtained and used as follows: Anti-HA monoclonal antibody (12CA5, Roche WB: 1:140 IF: 1:100); rabbit polyclonal anti-HA antibody (Y-11, Santa Cruz IF: 1:100); anti-PML mouse monoclonal antibody (PG-M3, Santa Cruz IF: 1:100); anti-SC35 mouse monoclonal antibody (S4045, Sigma IF: 1:100); anti-BrdU mouse monoclonal antibody (RPN202, Amersham Biosciences); anti-SUMO-1 mouse monoclonal antibody (GMP1, Zymed IF: 1:100); anti-SUMO-1 rabbit polyclonal antibody (SC-9060, Santa Cruz IF: 1:100); anti-Daxx rabbit polyclonal antibody (M-112, Santa Cruz IF: 1:100); anti-Flag mouse monoclonal antibody (M2, Sigma WB: 1:1000 IF: 1:1000); anti-V5 mouse monoclonal antibody (46-0705, Invitrogen WB: 1:1000); anti-His mouse monoclonal antibody (34610, Qiagen WB: 1:2000); anti-GST goat antibody (27457701, Amersham Pharmacia Biotech WB: 1:500); anti-βgalactosidase monoclonal antibody (Z378A, Promega WB: 1:5000); anti-GFP polyclonal antibody (SC-8334, Santa Cruz WB: 1:1000);

Secondary antibodies were used as follows. Anti-rabbit HRP (Sigma 1:1000), plus biotinylated anti-mouse and anti-rabbit antibodies (DAKO 1:1000) for western blots. Alkaline phosphatase conjugated goat anti-mouse (A-3562, Sigma 1:20,000) and alkaline phosphatase conjugated rabbit anti-goat antibody (A-4187, Sigma 1:20,000) for far western blots. Rhodamine conjugated goat anti-mouse (Molecular Probes 1:700), fluorescein conjugated goat anti-mouse (Molecular Probes 1:700), rhodamine conjugated goat anti-rabbit (Molecular Probes 1:700) and fluorescein conjugated goat anti-rabbit (Molecular Probes 1:700) for Immunofluorescence studies.

Production of GST fusion proteins in bacteria and in vitro GST-pull down assays

For protein production and purification, 40 ml of an overnight culture of *E. coli* strain DH5-α previously transformed with the appropriate expression plasmids were inoculated in a one to ten volume of Luria Broth containing ampicilin and grown at 37°C up to an OD of 0.6. Recombinant protein expression was induced for three hours with 1mM Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma). The cells were harvested by centrifugation, disrupted by sonication in lysis buffer (PBS, 1% Triton X-100, 100 U/ml DNAse, protease inhibitors cocktail, Calbiochem) and the lysates were then cleared from cell debris by centrifugation. The GST-fusion proteins were then incubated for one hour with glutathione-conjugated agarose beads (Sigma) at 4°C and bound proteins were washed extensively with lysis buffer. The levels and purity of proteins were determined by SDS-PAGE and Coomassie Brilliant Blue R (Sigma) staining.

For GST pulldown assays proteins were translated *in vitro* either in rabbit reticulocyte lysate or wheat germ extract respectively, using the Promega TNT system, and radiolabelled with [^{35}S]-cysteine (Amersham) according to the manufacturer's instructions. Approximately equal levels of *in vitro* translated protein were added to GST fusion proteins bound to glutathione resin, and incubated for 1 hr at 4°C. After extensive washing in PBS containing 0.5% NP40, the bound proteins were analysed by SDS-PAGE and autoradiography. For the peptide inhibition studies, E6 proteins were *in vitro* translated and than pre-incubated with different synthetic peptides (1 mM concentration) for 20 min at room temperature prior to the addition of GST fusion protein bound to the resin. Incubations were performed for 1 hr on ice. After removal of the supernatant, the resin was washed three times with PBS containing 0.25% Nonidet-P 40. Bound protein was eluted and analysed by SDS-PAGE and autoradiography. Quantitation of the levels of interaction was done by scanning the gels using either a Packard Instant PhosphoImager or a Cyclone (Packard).

Purification of His_6-HPV-16 E2

The *E. coli* DH5-α cells transformed with pQE9His_6-HPV-16 E2 were induced with 1mM IPTG for 3 hrs as described above. The cells were harvested by centrifugation and the pellet resuspended in lysis buffer (10 mM Tris-HCl pH 8.8, 300 mM NaCl, 0.1 M phosphate buffer pH 8.0, 100 U/ml DNAse). After sonication the cell debris was removed by centrifugation and the supernatant was incubated with Ni-NTA (Qiagen Inc.) for 1 hr at 4°C. The complexes were then washed and the bound protein was eluted with increasing amounts of imidazole. The purity of the protein was determined by SDS-PAGE and Coomassie Brilliant Blue R (Sigma) staining.

Far western blot

Purified GST fusion proteins of HPV-16 E6, HPV-16 E1, GST alone and BSA were run on SDS-PAGE and transferred to nitrocellulose. The membrane was blocked with milk buffer (PBS, 10% milk powder, 0.05% Tween 20) for 1 hr, washed, and incubated for 2 hrs at room temperature with the purified His-tagged HPV-16 E2 in milk buffer. After extensive washing, E2 was detected by incubating the membrane with a mouse anti-His (34610, Qiagen 1:2000) antibody for 1.5 hrs at room temperature. After further washing the blot was incubated with alkaline phosphatase conjugated goat anti-mouse (A-3562, Sigma 1:20,000). The converse experiment, where the bacterially expressed His-tagged E2 was transferred to nitrocellulose and incubated with the different GST fusion proteins was also performed. In this case goat anti GST-antibody (27457701, Amersham Pharmacia Biotech 1:500) followed by alkaline phosphatase conjugated rabbit anti-goat antibody (A-4187, Sigma 1:20,000) were used. Westerns were then developed using NBT/X-phosphatase solution (Boehringer Mannheim).

In vitro degradation assays

Proteins were *in vitro* translated using the TNT-coupled rabbit reticulocyte system (Promega) according to the manufacturer's instructions in the presence of either [^{35}S]-cysteine or [^{35}S]-methionine (Amersham). HPV-16 E6, HPV-18 E6 and p53 were under the control of SP6

promoter, whereas HPV-16 E2, MAGI-1, MAGI-2, MAGI-3 and Dlg were all under the control of a T7 promoter. *In vitro* translated E6 proteins were pre-incubated with each peptide (1mM concentration) or with *in vitro* translated HPV-16 E2 and either PBS or water-primed lysate as a control, for 20 min at room temperature prior to the addition of the *in vitro* translated target protein. Reaction mixtures were incubated at 30°C. Typically, p53 degradation assays were incubated for 30 min, MAGI degradation assays for 1 hr and Dlg assays for 2 hrs. Remaining target protein was immunoprecipitated using C4 polyclonal antibody for p53; a polyclonal rabbit antibody raised against the GST-NT.Dlg for Dlg and a polyclonal rabbit antibody raised against a GST-WW domain fusion protein for MAGI proteins. After extensive washing with E1A buffer (50 mM Hepes pH 7.0, 0.1% NP40, 250 mM NaCl) beads were eluted with Laemmli sample buffer, separated on SDS-PAGE gels, fixed in 10% acetic acid-30% methanol, dried and exposed to Kodak X-Omat film. All data were quantified on a Cyclone (Packard) or PhosphorImager and the percentage of degradation was calculated.

Where proteasome inhibitors were included in the assays, the proteins were translated *in vitro* in the presence of either 1mM proteasome inhibitor N-CBZ-leu-leu-leu-al (CBZ/MG132, Sigma) or DMSO as a control, and then used for degradation assays as above.

E6-AP depletion assay

Depletion of E6-AP was done by incubating *in vitro* translated proteins (10 µl) with polyclonal rabbit anti-E6-AP antibody (1 µl) for 30 min on ice followed by the adsorption of the immune complexes onto A-Sepharose beads (Amersham Biosciences). The resulting supernatant fractions were used as *in vitro* translated proteins depleted of E6-AP. The efficiency of the depletion was determined by autoradiography and quantification on a Cyclone (Packard). A pre-immune antibody was used as a negative control.

Peptide binding assays

In vitro translated radiolabelled HPV-16 and HPV-18 E6 proteins were mixed with the

biotinylated peptide P-5 (1 mM) and incubated for 20 min at room temperature. Proteins bound to the biotinylated peptide were recovered by the addition of 20 µl of avidin-agarose beads (Sigma). After extensive washing with the E1A buffer (50 mM Hepes pH 7.0, 0.1% NP40, 250 mM NaCl), bound proteins were analysed by SDS-PAGE and autoradiography.

Cells and transfections

U2OS (human osteosarcoma), Saos-2 (human osteogenic fibrosarcoma) 293 (human embryonic kidney) and HaCaT skin keratinocyte cells were grown in DMEM supplemented with 10% foetal calf serum. Transfections were performed using calcium phosphate precipitation as described previously (Graham & van der Eb, 1973) or by using lipofectamine (Invitrogen) as recommended by the manufacturer.

For the proteasome inhibition experiments cells were grown for 3 hrs either in the presence of either 50 µM N-CBZ-leu-leu-leu-al (CBZ/MG132, Sigma) or 10 µM N-acetyl-leu-leu-norleucinal (LLnL/MG101, Sigma) diluted in DMSO. DMSO alone was added as a negative control.

Immunofluorescence

Twenty-four hours after transfection U2OS and HaCaT cells were fixed in 3.7% paraformaldehyde in PBS for 20 min at room temperature. After further washing in PBS, the cells were permeabilized in PBS/0.1% Triton for 5 min, washed extensively with PBS, and then incubated with the primary antibody diluted in PBS for 1 hr. After 5 washes in PBS, the cells were incubated with a secondary antibody, as indicated, for 30 min. The cells were then washed several times in water and mounted on glass slides.

For the immunofluorescence analysis of protein association with mitotic chromosomes, the assys were done as described above with minor modifications. The U2OS cells were fixed 48 hrs after transfection and counterstained with 1 µM Hoechst (B2883, Sigma) before mounting on glass slides.

To determine the phase of cell cycle in U2OS cells 5'-bromo-2'-deoxyuridine (BrdU) incorporation was performed. 24 hrs after transfection, the cells were pulse-labeled for 1 hr with BrdU, fixed and subjected to a denaturation step with 0.05 M NaOH in PBS for 10-20 sec. The cells were then washed several times in PBS and incubated with anti-BrdU mouse monoclonal antibody (RPN202, Amersham Biosciences).

Cells were visualised by using either a Zeiss Axiovert 100M microscope attached to a LSM 510 confocal unit or a Leica DMLB fluorescence microscope equipped with a Leica photo camera (A01M871016).

Western blotting

For E6 detection, U2OS cells were transfected and harvested after 24 hrs. Cells were washed once in ice-cold PBS and then scraped in high-salt E1A buffer (50mM Hepes pH 7.0, 0.1% NP40, 500 mM NaCl plus a protease inhibitor cocktail, Calbiochem). Cells were sonicated for 5 sec and then left on ice for 20 min. The insoluble fraction was separated from the soluble fraction by centrifugation at 13,000 rpm for 10 min. Following SDS-PAGE, the extracts were blotted on a 0.22 μm nitrocellulose membrane (Schleicher & Schuell) and then incubated with the appropriate primary antibody in 1% milk/0.5% Tween 20 for 1.5 hrs. 12CA5 (1:140, Roche) was used to detect HA-tagged proteins, rabbit anti-E2 (1:500, Massimi et al., 1999) to detect E2 and anti-β-galactosidase (1:5000, Promega) to detect β-galactosidase. Blots were developed using the Amersham ECL technique according to the manufacturer's instructions.

For the detection of p53, Dlg and MAGI proteins, the cells were rinsed in ice-cold PBS, lysed on ice in buffer E1A (50 mM Hepes pH 7.0, 0.1% NP40, 250 mM NaCl plus a protease inhibitor cocktail, Calbiochem) for 20 min and then cleared by centrifugation at 13,000 rpm for 10 min at 4°C. Extracted proteins were separated on SDS-PAGE and transferred to 0.45 μm nitrocellulose membrane (Schleicher & Schuell), detected with the appropriate specific antibodies and developed with the Amersham ECL System according to the manufacturer's instructions.

Immunoprecipitations

U2OS cells were transfected with either pCMV-E2 alone or in combination with Flag-tagged wild type or mutant HPV-18 E6. After 24 hrs the proteins were extracted as described above and the soluble fraction was diluted down to a final salt concentration 250 mM NaCl and than incubated for 3-4 hours with the 40 µl of anti-Flag agarose beads (M2 Sigma) on a rotating wheel at 4°C. After three washes in 250 mM NaCl E1A buffer the eluted beads were subjected to SDS-PAGE followed by western blotting. Coprecipitated E2 was detected by using an anti-E2 polyclonal antibody.

In vivo degradation assays

p53. Saos-2 cells were transfected with 4 µg of the p53 expression construct, together with 5 µg of HA-tagged HPV-16 and HPV-18 E6 alone or in combination with 5 µg of HPV-16 E2 expression plasmid. 0.5 µg of CMVLTR-LacZ expression vector was also included to allow normalisation of different experiments for transfection. Alternatively, U2OS cells were transfected with 5 µg of the Flag-tagged p53 expression vector and with either 1, 3 or 6 µg of HA-tagged HPV-18 E6 expression vectors, together with 3 µg of GFP expression vector to allow normalisation for transfection efficiency. 24 hours post-transfection, the cells were harvested and analysed by western blotting as above described.

MAGIs. U2OS cells were transfected with 5 µg of the appropriate MAGI expression vector together with 5 µg of HA-tagged HPV-16, HPV-18 E6 or the HPV-18 E6 ΔM mutant alone or in combination with 5 µg of HPV-16 E2 expression plasmid. 0.2 µg of CMVLTR-LacZ or 3 µg of GFP expression vectors were also included to monitor transfection efficiency.

RT-PCR

U2OS cells were transfected with 10 µg of the HPV-18 E6 expressing vector alone or together with CMV-HPV-16 E2 plasmid. 3 µg of GFP expression vector was also included to monitor

transfection efficiency. 24 hrs after transfection total cellular RNA was extracted using TRI REAGENT (Sigma) according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed using RETROscript system (Ambion). HPV-18 E6/E6*I and GFP were then amplified by PCR through 30 cycles. Primers used are as follows:

Forward GFP: 5'-CGTAAACGGCCACAAGTTC-3'

Reverse GFP: 5'-GGCGGATCTTGAAGTTCAC-3'

Forward HPV-18E6: 5'-ATGGCGCGCTTTGAGGAT-3'

Reverse HPV-18 E6: 5'-TTATACTTGTGTTTCTCTGCGTCGTTG-3'

Reverse HPV-18 E6*I: 5'-GCACCGCAGGCACTCTGTAAGTTCCAA-3'.

Amplification conditions were: 30 sec at 95°C, 30 sec at 55°C for GFP or 60°C for HPV-18 E6/E6*I and 30 sec at 72°C. Products were visualised following agarose-gel electrophoresis.

Dual luciferase reporterassay

Transfections were carried out in Saos-2 cells using lipofectamine (Invitrogen) in 6-well plates according to the manufacturer's instructions. Cells were transfected with 6xE2BS-Luc (0.5 μg) and one or more of the following, CMV-16 E2 (0.5 μg) and pcDNA-16 E6 Δ123-127 (0.5 μg) as indicated. Total amount of DNA was equalised with empty plasmids. All transfections were carried out in duplicate and repeated at least three times. Simultaneous expression of pRL plasmid provided an internal control of baseline response and allowed for the normalisation of transfection efficiency. 24 hours post transfection, cells were lysed and luciferase activity was measured using the Dual-luciferase assay kit as recommended by the manufacturer (Promega).

Gel retardation assay

Oligonucleotodes corresponding to two E2 recognition sites, 5'-GCTTCAACCGAAATCGGTTGAACCGAAACCGGTTGCATG-3' were hybridised by heating for 5 min at 65°C in hybridisation buffer (0.5 M NaCl. 0.5 M Tris pH 8.0) and then left to cool down slowly to room temperature. Hybridised oligonucleotides were then labelled in the

presence of [γ^{32}P]ATP (Amersham) and polynucleotide kinase. Gel shifts were carried out by mixing the probe (40000 cpm, 100 ng) with the specific proteins expressed *in vitro* using the TNT coupled transcription-translation system (Promega) in a buffer containing 20 mM Hepes pH 7.9, 50 mM KCl, 2 mM DTT, 10 mM $MgCl_2$, 1% glycerol and in the presence of 20 ng of non-specific competitor DNA (polydIpolydC, Amersham Biosciences). After incubation at room temperature for 30 min, loading dye was added and the samples were resovled on non-denaturing 6% acrylamide (acrylamide:bis, 40:1) gels in 0.5X Tris-borate-EDTA. The gels were then dried and processed by autoradiography.

For supershift analysis 1 µl of the polyclonal anti-E2 antibody raised against the C-terminal domain of the E2 protein was added to the E2-oligonucleotide complex after the former 30 min binding reaction and the mixture incubated for a further 30 min on ice. As a control 1 µl of rabbit preimmune was used.

Transient DNA replication assay

Transient DNA replication assays were performed in 293 cells transfected by the calcium phosphate method with 1 µg of the pCGE1BΔE5 vector, expressing HPV-16 E1 and HPV-16 E2, together with 3 µg of the replicon p16 URR::CAT and 6 µg of the E6 expressing plasmids. Three days posttransfection, low-molecular-mass DNA was isolated by the Hirt extraction procedure (Hirt, 1967). Samples were digested overnight with HindIII to linearize the *ori* plasmids and treated with DpnI to remove the unreplicated input methylated DNA. Total digestion products were separated on a 0.8% agarose gel in Tris-acetate-EDTA buffer. The DNA products were transferred to a Hybond-N^+ membrane (Amersham), and subsequently hybridized to a ^{32}P-labeled replicon probe generated by random priming. Blots were hybridized overnight at 42°C in a solution containing 6x SCC, 5x Denhardt's solution, 0.5% SDS, 50% formamide and 500 µg/ml herring sperm DNA. Blots were washed twice for 30 min at 42°C in 2x SSC and 0.1% SDS and then twice for 30 min at 55°C in a solution containing 0.2x SSC and 0.1 SDS. Blots were processed by autoradiography.

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